OSTEOPETROSIS AND THE LEUCOSIS COMPLEX

By

DAVID YOUNG

Submitted to the University of Edinburgh as a Thesis in fulfilment of the requirements for the degree of Doctor of Philosophy.

Poultry Research Centre,
EDINBURGH.

NOVEMBER 1964.

CONTENTS

SECTION I

GENERAL INTRODUCTION

The current interest in oncogenic viruses is based on the original findings of Rous and Murphy (1912), who were the first to show the aetiological relationship of a virus with a tumour, and, more recently, the work of Gross (1950) who first demonstrated virus-induced leukaemia in A.K. mice. This discovery has been followed by the work of Friend (1957), Moloney (1960), Schwartz et al (1956), and Graffi (1957), all of whom demonstrated virus-induced leukaemias. The accumulation of evidence from the extensive studies of conditions associated with viruses has revealed more and more examples of viruses intimately concerned with the development of cancer. Studies on human cancers have so far failed to show an aetiological relationship with virus particles (Bryan, 1962), but there are several recent reports of the presence of virus particles in tissues from cancer patients, (Dmochowski, 1963; Almeida et al, 1963; Epstein, 1964). Birkett's suggestion (1963) that an insect vector may be responsible for the spread of a lymphoid tumour, infers that viruses may be responsible for human cancers. Comparative studies in other animals have been used as models in order to study virus-induced leukaemias, and the viruses responsible for avian leucosis were amongst the first to be examined, (Engelbreth-Holm and Rothe-Meyer, 1932; Rothe-Meyer and Engelbreth-Holm, 1933; Stubbs and Furth, 1935; Hall et al, 1941; Burmester et al, 1946).

Unfortunately, the position of avian leukaemic viruses in the general classification of virus-induced conditions is rather confused. This may be due to the now accepted difficulties in isolating and cloning viruses with a view to obtaining pure strains (Hanafusa, 1963). Before considering the implications of the behaviour of the avian leukaemic viruses, a few examples of the isolation procedures, and proofs of causal relationships for a few selected viruses may help as comparisons.

The growth characteristics of polyoma virus are good examples of the complexity of virus behaviour, resulting in the difficulties encountered when attempts are made at rationalisation. Polyoma virus was first reported by Gross (1957), who had initially shown that cell-free extracts from leukaemic A.K. mice, induced leukaemia when inoculated into newborn C_3H mice. Fibrosarcomata and parotid tumours were also induced by these extracts. Gross demonstrated that there were two separate viruses involved in his original passaged material; one responsible for the leukaemia, and the other for the solid tumours. This second virus, initially referred to as the parotid tumour agent, was successfully isolated by Stewart et al (1957a,b), using tissue culture techniques, and the distinction between polyoma and the leukaemic agent was confirmed by Negroni et al, (1959); Merekalova (1961); Gimmy and Graffi (1961). McCulloch et al (1959) point out that a virus identical to polyoma was isolated from a spontaneous mouse mammary tumour, which was not transplantable by cell-free filtrates. This type of association between a virus which is not apparently the causal

agent of the tumour, is frequently reported (Negroni, 1963). Another typical example of this type of association is reported by Friend (1957), who isolated a virus from an Ehrlich's ascites tumour of a Swiss mouse. The tumour cells could be transplanted from mouse to mouse (Friend and Haddad, 1960) producing ascites tumours, but cell-free filtrates produced leukaemia in adult mice shortly after injection. Like Friend's virus, the Moloney virus (Moloney, 1960) was also isolated from mouse sarcoma (37) cells, although in this case the tumour had been passaged for many years. Similarly, the virus responsible for Graffi's disease (Graffi, 1957) was also isolated from a transplantable Ehrlich's ascites tumour.

These few examples show that the mere presence of a virus is not alone acceptable as a criterion of the unequivocal identification of an agent as the specific tumour virus (Haguenau, 1960a,b; Haguenau and Beard, 1962). On the other hand, human adeno-virus (Trentin et al, 1962), which was considered a non-tumour inducing virus, produced tumours of lung, thorax and liver when injected into new-born hamsters. These experiments show that viruses which do not produce tumours in one host may exhibit oncogenic characteristics in another. It is thus tempting to extrapolate, and suggest that these same viruses may occasionally be responsible for neoplastic growth in their primary host. However, in the field of human cancer research, there has not yet been a convincing report of the isolation of a tumour inducing virus from a human tumour, although Ageenko (1960), Huebner (1961) and many others have emphasised that it

is impossible to exclude the possibility of viral causative agents in human cancers. Viruses which are associated with tumours, but which are not aetiologically related, have been called passenger viruses (Negroni, 1963). This class of virus includes some of the leukaemia viruses previously described, and also some non-oncogenic types, whose presence further complicates the demonstration of cause and effect between a particular tumour and a virus. Toolan's virus (Toolan, 1954), Gallus adeno-like (G.A.L.) (Stoker, 1959), and Riley's agent (Riley and Wroblewski, 1960), are typical of viruses whose presence in tumour tissue appears to be solely fortuitous.

It now appears that passenger viruses must be regarded as the rule, rather than the exception, when examining tumour tissue. The dangers inherent in this type of situation have recently been emphasised by the investigation of two human conditions, Birkett's lymphoma (Birkett, 1963; Epstein, 1964) and human myeloid leukaemia (Negroni, 1964; Inman et al, 1964). In both these conditions, association with viral agents has been shown, but without the convincing evidence necessary to prove aetiological relationships. In fact, Birkett's lymphoma has been passaged in tissue culture, through several generations, and the cultured cells now appear to contain at least nine recognisable viruses, including herpes simplex and strains of reo virus (Epstein, 1964).

With this possibility of purely accidental associations of virus particles, it is not surprising the aetiological relationships of any one particular group of viruses are confused.

The viruses responsible for the avian leucosis diseases have

been the objects of much conjecture, resulting from the uncertainty as to whether or not the viruses under investigation formed homogenous populations. The leucosis group consists of three basic types of leukaemic conditions, but the distinctions between the different types is not as clearly defined as in some other animal virus systems. Consequently, rather than 'pure' lines of virus being available, 'strains' of leucosis viruses have been isolated, each of which has its own typical disease pattern. Isolated 'strains' are summarised in the table on page 12.

Evidence for a viral cause of the avian leucoses has been well established, but the aetiological relationships between different groups of symptoms have remained obscure due to the variations in host response, even within a virus strain. As a result of this spectrum of responses, the explanations have ranged from the concept of individual entities being responsible for each condition (Jungherr, 1952), to the idea that a single viral entity can induce all the leucosis conditions (Burmester, 1962). Further confusion stems from the reports that both osteopetrosis (Burmester, 1946) and fowl paralysis (Marek's disease) (Jungherr and Landauer, 1938) have been encountered in experiments involving the passaging of these leucosis conditions. These two conditions do not strictly come within the definitions of leucosis, and several authors (Campbell, 1961; Biggs, 1961) have argued that osteopetrosis and fowl paralysis should not be regarded as conditions that can be induced by the 'leucosis complex' of viruses. It would appear from a study of the literature that some of the reasons for the confusion are due to the following factors.

First, a clear account of the life cycles and natural propagation of the leucosis viruses in cells and tissues has not been described as comprehensively as those of many of the mammalian viruses, e. g. polyoma, polio and influenza. In fact, the agent responsible for lymphomatosis has yet to be described, even though several authors (Dmochowski, 1959) have observed particles in the spleen of infected birds.

Secondly, workers have been using strains of virus (Beard, 1963) isolated from birds carrying simultaneous infections of other diseases, (see table 1, page 12). As a result, there is no guarantee that these are homogeneous populations of viruses. On the contrary, since many of the workers in the field of avian viruses work in the United States, where there is a high natural incidence of leucosis (Burmester, 1947; Baluda, 1962), it is possible that contamination of strains of virus might occur in routine passaging, if not in the original isolation.

Thirdly, many of the reported characteristics of the leucosis viruses have been based on experiments involving the transplantable lymphoid tumour R.P.L. 12 (Olson, 1941; Burmester et al, 1946), which has been passaged in chickens for 23 years. In the light of present knowledge of passenger viruses, it seems possible that the continuous passage of a tumour in chickens, known to have a high natural incidence of leucosis, for a period as long as this, would almost inevitably lead to contamination of the tumour with aetiologically unrelated viruses.

Finally, although both osteopetrosis and fowl paralysis have been reported to be induced by the viral strains of leucosis

mentioned, (Burmester et al, 1946), the causative agents have not been identified and characterised. Consequently, on the suggestion of Dr. John Carr, an attempt has been made to characterise the causative agent of osteopetrosis and elucidate its life cycle, in the hope that the relevance of the reported association of osteopetrosis with the avian leucosis viruses could be critically discussed.

Several authors, (Jungherr and Landauer, 1938; Holmes, 1961; Campbell et al, 1964), have described osteopetrosis and the associated tissue involvement in considerable detail: a summary of the pathology is therefore now included in order to show the basis of the work reported in this thesis.

Recent findings by Campbell, Young and Carr show that, in advanced cases of osteopetrosis, the lesions and tumours most commonly encountered involve the bones, reticulo endothelial system, striated muscle and kidneys. The lesions first observed macroscopically consist of a periosteal thickening and ossification, which is followed by an endosteal proliferation and conversion to bone. This results in the eventual obliteration of the medullary cavity and the appearance of myelophthysic anaemia; on the other hand, there is no recognisable leukaemia.

The spleen is frequently atrophied and shows, histologically, a marked depletion of the white pulp and patches of hyaline, which are not invariably associated with vessels.

The liver, especially in females, shows, initially, a peculiar endotheliosis, involving a thickening of the Kupffer cell fibres, and an atrophy of the liver cell cords, producing an

unusually prominent space of Disse. At a later stage, some cirrhotic changes occur together with biliary hyperplasia, and foci of intramedullary haematopoiesis.

The gonads in experimentally infected birds are frequently found, at post mortem examination, to be juvenile and comparable to those of four to six week old chickens. Branches of the ovarian and testicular arteries within these organs frequently show great hypertrophy of the muscle walls unassociated with any degenerative change.

In a proportion of cases affected, male birds have been found to have flattened or spindle shaped fibrous lesions, commonly situated in the gracilis or sartorius muscle, or, less commonly, in the pectoral muscle. It is rare to find these muscle lesions in females. Histologically, these lesions initially consist of a disorientated proliferation of muscle cells which are intimately associated with adjacent normal muscle bundles. Later, the lesions show fasiculi of mesenchymal cells, fragments of muscle fibres, acidophilic cells, a good deal of fibrous tissue, and fairly extensive plasma cell infiltration.

Kidney abnormalities have been described, and can be divided into three categories.

1. Proliferative glomerular lesions, which occur in the majority of experimental birds, even when the bones are not noticeably affected.

2. Tubular hypertrophy and cysts which are frequently found in subcapsular regions. The tubules involved are those comprising the first loop of the collecting ducts, and possibly

part of the ascending limb of the loop of Henle. The cysts are lined with flattened epithelium, whereas the hypertrophied tubules have a cuboidal epithelium, which is occasionally ciliated. This type of abnormality appears to be mainly confined to the male.

3. Tumours of two histological variants have been found: one is a typical embryonal growth of the Wilms variety (nephroblastoma), the other appears to be associated with renal cysts and has been described as "a clear-cell carcinoma of renal origin". Tumours have also been reported in the liver and endocrine organs, although it should be emphasised that these tumours have a relatively low incidence (see table 2, page 33), but in one group of birds injected with frozen, dried blood (Campbell et al, 1964), kidney tumours were much more common than in birds infected with fresh blood.

Transmission of Osteopetrosis. Experiments demonstrating a transmissible agent responsible for this disease were first reported by Jungherr and Landauer (1938). These workers showed that osteopetrosis could be transmitted by whole blood, bone marrow or tumour tissue from birds diagnosed as having neurolymphomatosis, as well as osteopetrosis. Inoculation of these tissues resulted in the passage of osteopetrosis and lymphomatosis, and the two conditions were claimed to be 'non-dissociable'. The transmissible agent present in the blood, bone marrow and lymphomata, survived dessication for 105 days. More recently, Holmes (1958) succeeded in transmitting the disease from one of three spontaneous cases of osteopetrosis occuring in a small

flock of about one hundred fowls. Day-old chicks, injected intraperitoneally with whole blood marrow and filtered plasma, developed the disease. Holmes (1958) also found that inoculation of infected whole blood onto the chorio-allantoic membrane also caused osteopetrosis later in the hatched chicks.

He claimed that the infection in the egg may be one mode of transmission. Subsequently, Holmes (1959) was to show the filterability of the agent, its probable presence in the semen of infected male birds, and the infectivity of unfiltered plasma when administered orally. Holmes (1961), in a later study of osteopetrosis, concluded that the transmissible agent had an affinity for parenchymatous tissue, but that the infective agent should not be considered as part of the avian leucosis complex, since the associated soft tissue changes were not typical of leucosis. Holmes (1963) has reported the transmission of osteopetrosis from chickens to young turkeys.

The conflicting reports from the United States (Burmester et al, 1946-63) (table 1, page 12.), will be considered in detail in Section VI. However, since the present work has been concerned with the virus isolated in this country (Holmes, 1958), the characteristics of this strain only have been considered. The virus transmitted by Holmes, in Bristol, and more recently at the Poultry Research Centre, has been regarded as a strain of osteopetrosis on the same grounds as the leucosis conditions mentioned on page 5 of this thesis. This United Kingdom strain of virus has been maintained for some time in flocks of chickens in which leucosis has not been observed.

The flock at the Poultry Research Centre (Greenwood and Carr, 1951; Campbell, 1963a) is considered to be free from leucosis, and since no case of leucosis has arisen in birds injected with the osteopetrosis preparations, the assumption has been made that the virus 'strain' is, in fact, free from leucosis producing agents.

Since the gross pathology of the disease has been adequately reported, the experiments reported in this work have been directed towards the characterisation of the virus, rather than the effect on the infected cells. On these assumptions, a series of experiments has been carried out, which aimed at showing

1. that osteopetrosis is, indeed, transmissible by a virus;
2. the character of the virus;
3. the behaviour of the virus in vitro, with a view to developing an assay method;
4. the life cycle;
5. the relationship, if any, of osteopetrosis with the leucosis complex and the transmissible R.P.L. 12 lymphoid tumour.

TABLE 1

STRAINS OF LEUCOSIS VIRUSES MAINTAINED IN EXPERIMENTAL BIRDS

Original leucosis type	Designation	Original Reference	Conditions which have been associated with the strain
Erythroleucosis	Erythroleucosis Strain R	Engelbreth-Holm and Rothe-Meyer (1932)	1935-55 1,255 cases of leucosis, 1 myeloid leucosis, 9 mixed erythro and myeloid leucosis 1955-63 only erythroleucosis
Myeloid leucosis	Erythroleucosis Strain E.S. 4	Rothe-Meyer and Engelbreth-Holm (1933)	1933-35 684 cases of erythroleucosis, 194 sarcoma, 188 erythroleucosis and sarcoma, 3 myeloid leucosis with sarcoma 1956 8 cases erythroleucosis with renal carcinomas
Fowl paralysis (Mareck's Disease)	Myeloid leucosis B.A.I. Strain A	Hall et al (1941)	1941-43 Strain 1 56% birds inoculated developed erythroleucosis 42% developed lymphoid leucosis Strain 2 lymphomatosis, erythroleucosis, myeloid leucosis 1952-63 only myeloid leucosis
Lymphoid leucosis	R.P.L. 12 (lymphoid leucosis)	Olson (1941) Burmester (1946)	1941-46 Solid lympho-sarcoma 1946-60 Lymphoid leucosis, osteopetrosis 1960-63 Lymphoid leucosis, osteopetrosis, erythroleucosis, (gallus adeno-like virus)
Lymphoid leucosis	Strain 13 (Sarcoma associated with erythroleucosis)	Stubbs and Furth (1935)	1931-35 Myeloid leucosis, erythroleucosis, lymphoid leucosis, myeloma

SECTION II

DEMONSTRATION OF A TRANSMISSIBLE AGENT ASSOCIATED WITH OSTEOPETROSIS

INTRODUCTION

The original sample of osteopetrosis infected blood came from Dr. J. R. Holmes of the College of Veterinary Surgery, Bristol University, in 1958. Dr. Holmes obtained the blood from a field case of spontaneously occuring osteopetrosis, diagnosed by himself, and thought to be free from leucosis. The condition had been maintained by him by serial passage of infected blood into day-old chickens, and a specimen from these has been kept at the Poultry Research Centre now, over a period of six years. A summary of the routine passage experiments has been included in table 2 on page 33 to give an indication of the constancy of the features of this maintained strain. Associated tumours are recorded in the control, as well as the osteopetrosis infected birds: the controls in this case being the whole flock of Poultry Research Centre birds. It should be noted, although this point will be discussed later, that at no time has there been a case of spontaneous virus-associated transmissible leucosis in the flock. Consequently, it has been assumed that the original sample of blood, obtained from Dr. Holmes was not contaminated with leucosis virus, and that the leucosis viruses are completely absent in this flock of birds, (Campbell, 1963a; Greenwood and Carr, 1951).

From this table it can be concluded that the characteristics of the osteopetrosis condition maintained in this

laboratory were similar to those reported elsewhere, (Jungherr and Landauer, 1938; Pugh, 1927; Holmes, 1958). This artificially maintained pathological condition was probably identical, in all respects, with spontaneous cases.

It is now generally accepted (Jungherr and Landauer, 1938; Burmester et al, 1946; Holmes, 1958; Bell and Campbell, 1961; Campbell et al, 1964), that osteopetrosis is associated with some type of transmissible agent. However, neither the nature of this transmissible agent, nor its method of infection, have been described. Consequently, an initial series of experiments was undertaken to determine the approximate size of the infective agent, by filtration through graded millipore filters.

MATERIALS AND METHODS

Blood from infected birds was obtained with a hypodermic syringe from a vein in the wing, using citrate as an anticoagulant. This blood was then centrifuged at 3,000g. to remove all the blood cells. The plasma was divided into three parts, and each fraction was filtered through one of the following three types of millipore filters.

Type H.A. with a pore size of 4,500Å ± 200Å.

Type G.S. with a pore size of 2,200Å ± 200Å.

Type V.C. with a pore size of 1,000Å ± 80Å.

The filtered plasma was then injected in 0.2 ml. doses into day-old male chicks, and the birds were then examined for a period of approximately fourteen to sixteen weeks, before being killed and autopsied. These birds were then compared with

chicks injected with 0.2 mls. of unfiltered and infective whole blood.

RESULTS

Treatment of blood	birds developing osteopetrosis Number injected
Filtered with H.A. (4500Å pore size)	3/9
Filtered with G.S. (2200Å pore size)	2/8
Filtered with V.C. (1000Å pore size)	0/10
Unfiltered plasma	3/10
Whole blood	190/335
Non-infected, unfiltered	0/whole flock

(The "whole blood" figures in this table are composed of the results of routine passages and other large scale experiments.)

These are extremely small numbers of birds and are consequently of little statistical significance. However, the difficulty of keeping large numbers of birds for long periods was such that only small numbers could be kept as adults. Even so, the plasma filtered with G.S. and H.A. filters shows a reduction in infectivity - as suggested by Holmes (1958). The V.C. filtered fraction appears to be non-infective. Consequently, it was tentatively assumed that a particle of a size larger than 1,000Å was necessary for the development of osteopetrosis.

ELECTRON MICROSCOPE SURVEY

INTRODUCTION

The filtration experiment suggested that the blood contained a particle, greater than 1,000Å and less than 2,200Å in diameter, which was the causative agent of osteopetrosis. In order to determine whether or not such a particle existed, an electron microscope survey was undertaken. If such a particle was demonstrable, the electron microscope would readily show where such particles were concentrated, the general distribution in the different tissues of the bird, and the cellular involvement. Previous accounts - as described in the introduction - have indicated the lesions most commonly encountered in cases of osteopetrosis and so the tissues most frequently reported to be affected were examined.

Obviously, the blood is the first place to look for particles, since it is from here that sources of infective, transmissible cell-free material can be obtained. The next most likely site is the bone lesion, since this is the diagnostic feature of osteopetrosis. Periosteal cells are the ones most likely to be infected, since it is due to the hyperplastic condition of this layer of cells that the thickened bone results: osteopetrosis is not a true neoplasia, since the basic bone structure is retained, although somewhat less regular than normal, (Jungherr and Landauer, 1938). Muscle lesions and kidney abnormalities are often reported in osteopetrotic birds and were considered to be likely sources of particles (Campbell et al, 1964). The kidney is frequently a centre of virus replication,

and many different viruses have been reported as growing in this organ. Blood cells were examined in the spleen, since it was much easier to examine solid tissue than pellets of free cells. The spleen was also considered a likely place in which to find concentrations of any particles free in the blood, since this organ contains many phagocytic cells. The liver, too, contains a considerable number of reticulo-endothelial cells in the form of Kupffer cells: this organ, too, was examined. Finally, due to Holmes' suggestion (1958) that osteopetrosis can be transmitted vertically, from parent to offspring, and since the reproductive organs of both the male and the female (but particularly the male) are usually very small and juvenile, the ovary and testes of infected birds were examined.

MATERIALS AND METHODS

The details of procedures and the composition of stains, fixatives and embedding materials, are included in the Appendix to this thesis.

Microscope. The microscope used in this work was a Phillips E.M.100 which, with a pre-aligned lens system, a fixed bias, a single condenser and no stigmator to adjust the inherent astigmatism, is a relatively low resolution machine. The practical working resolution is approximately 25Å. The only modification made to the instrument was to instal a grid holder, similar to that used in the newer Phillips E.M.200 microscope. This was done in order to reduce the considerable amount of specimen drift which occured with the original holder.

Micrographs were taken using Ilford 5B-11, 35 mm. nuclear

track recording film, developed in I.D. 20 and printed by standard printing techniques. One of the difficulties with this microscope was due to the recording on 35 mm. film. This necessitated enlarging the negative five to six times when prints were made, resulting in a considerable loss of resolution in the enlargements. This is an inherent fault in the design of this machine, and accounts for the characteristic texture of the micrographs.

Grids. Athene type New 200 and 400 mesh copper grids were used filmed, initially, with formvar by the technique described by Bradley (1961), and stabilised with a carbon film evaporated in an A.E.I. type 12 Shadow Casting Unit. Occasionally, for higher resolution work, New 400 grids, without plastic films, were used to support thin sections. In these cases, the grids were first cleaned in chloroform to remove any contaminating grease.

Fixation. All tissues which were fixed for electron microscopy were obtained from birds which had been killed, and then dissected as quickly as possible. Pieces of tissue 2-3 mm^3 were removed and placed in the fixative, or, in the case of the periosteum, the bone was bathed in the fixative as soon as the muscles had been laid aside, before excision of the periosteum and fixation. In all cases, the fixative which was chosen for a particular tissue examination was previously cooled to 4°C. in a refrigerator, and the actual fixation carried out in small test tubes at 4°C.

Osmium Tetroxide. Palade's buffered osmium tetroxide (Palade, 1952) was used initially as a fixative, and gave quite good

results (Appendix, page XIII), but occasionally large areas of the fixed tissue appeared to be badly preserved: ribosomal and small particular constituents of the cytoplasm frequently appeared to be badly preserved, or even missing completely. It was thought that isotonic factors may have been causing osmotic shock to these fine structures, and so two other OsO_4 fixatives, suggested by Zetterqvist (1956) and Caulfield (1957) were tried, (Appendix, page XIII). These gave greatly improved preservation, particularly Caulfield's fixative, which included sucrose: this did not appear to precipitate the fibrillar and granular materials as coarsely as the other OsO_4 fixatives.

Luft's Buffered Permanganate. Luft (1956) recommends this fixative (Appendix, page XIV) for the preservation of membrane structures in cells, and coupled with embedding in epoxy resins (Robertson, 1958), this fixative was found to give reasonable preservation of these structures. However, permanganate gave very poor preservation of many non-membrane, cytoplasmic structures, and nuclear details were either poorly preserved or lost in the fixation process. This fixative was not used after the first trial experiments.

Glutaraldehyde, (Sabatini et al, 1962). This aldehyde fixative was found to be the best one tried for electron microscopy, (see page XIV of the Appendix). The preservation of submicroscopic structure was found to be excellent, when compared with that of material fixed in Caulfield's fixative. Furthermore, Sabatini claims that the activity of the enzymes aliesterase, acetylcholinesterase, alkaline phosphatase, acid phosphatase, 5-nucleotidase, adenosine triphosphatase, D.P.N.H. and T.P.N.H.

diaphorase can all be detected after fixation with glutaraldehyde. The best technique for determining enzymic activity after fixation, involves cutting frozen sections of the material and then examining the tissue by histochemical means. Little or no damage is recorded after frozen sectioning or histochemical incubations on subsequent examination in the electron microscope. The post-incubation fixation in osmium tetroxide, necessary to produce sufficient contrast in the electron microscope, allows the final product of the histochemical reactions to be related to fine structural elements as they normally appear. Obviously, tissues treated in this way are suitable for examination in the light microscope, as well as in the electron microscope. In comparisons of this sort, the glutaraldehyde fixed material shows little degeneration of cytoplasmic structures.

One final advantage of glutaraldehyde fixation is in the non-critical length of time necessary for good and complete fixation. Because of this, material can remain over-night in the fixing solution, which can be extremely useful.

Dehydration. Standard methods of alcohol dehydration were used, and are reported in the Appendix.

Embedding. Much time can be spent in finding the best embedding material and procedure for a particular tissue, and, in all the variations of embedding techniques tried, the aim was to produce quick, easy, reproducible methods.

Araldite. Since araldite (Glauert and Glauert, 1958) seemed to give the most reproducible results, and did not need a lengthier

preparation than the others, this epoxy resin was adopted for most routine preparations. Embedding with araldite appears to be improved if the final dehydration is carried out with epoxy propane as a vehicle (Durand, 1961). This final step helps penetration, since the araldite components are so viscous that, although alone they penetrate tissues very slowly, aided by a highly volatile material, with which they are miscible, penetration is considerably speeded up.

Other embedding materials used include:

Methacrylates. Initially, various mixtures of methyl and butyl methacrylates were used for embedding (Newman et al, 1949). There are several advantages in using methacrylate mixtures for embedding:

1. the monomers are fluids with low viscosities and are readily soluble in ethanol:

2. they polymerise either by heating at about 60°C, or by irradiation by ultraviolet light:

3. the hardness of the final block can be adjusted by varying the relative quantities of the two monomers. Consequently, the methacrylate blocks are very easy to section.

Unfortunately, there are several disadvantages in this material.

1. The polymerisation is usually uneven, so that polymerisation damage, due to uneven curing of the polymer, often results. This damage takes the form of disrupted tissues or of small bubbles of gas, which are left in the hardened blocks: shrinkage during this process can be as much as 20% of the

original volume.

2. Methacrylate sections are unstable in the electron beam and as much as 40% of the polymer in the section is lost immediately the electron beam is switched on, (Cosslett, 1951).

3. The vapour is harmful and unpleasant.

Consequently, since the disadvantages outweighed the advantages, this embedding material was discarded.

Durcopan. This water soluble embedding material was tried, since it was considered possible that it might be useful for enzymic extractions. Unfortunately, great difficulty was encountered in persuading this material to polymerise and harden sufficiently well to cut sections. No satisfactory sections were cut from blocks of this material.

Maraglas, (Freeman and Spurlock, 1962). This material, which is an epoxy resin, is reputed to be as good as araldite (or the American equivalent, Epon) in most respects, but with the added advantage that it is easier to cut. Only a few specimens have so far been prepared with this material, but initial experiments have shown it to be easy to cut, although preservation has not been as good as araldite: more tissues are being examined using maraglas, since an epoxy resin, giving reproducible results like araldite, but which is easily sectioned, would be a great improvement.

Stains. Various electron microscope stains were used on sectioned material, but by and large, the procedures recommended in the standard references were adhered to. A summary of the stains used, including the general properties of each stain, is given overleaf.

Stain	Reference	Results
Lead citrate	Reynolds (1963)	Very good, little non-specific precipitation
Lead tartrate	Millonig (1961)	Slow to penetrate
Lead hydroxide	Karnovsky (1961)	Quite good, considerable non-specific precipitation
$KMnO_4$ 2%	Luft (1956)	(Same as fixative) Good for low magnification
Uranyl acetate in 50:50 water:alcohol	Gibbons and Grimstone (1960)	Very good if kept out of sunlight and used fresh
Uranyl acetate in 100% alcohol	No reference	This solution extracted material from sections
Phosphotungstic 10%	Watson (1958)	Added little to contrast of sections
Phosphomolybdic acid 10%	Watson (1958)	Added little to contrast of sections

The best sequence of stains was found to be a twenty minute staining with a completely CO_2-free solution of lead citrate - as recommended by Reynolds (1963), followed by ten minutes in a 50:50 alcohol and water mixture, saturated with uranyl acetate.

The details of the non-standard procedures are included in the Appendix.

Pathological Material. Tissues for this general survey were obtained from chickens of the Brown Leghorn line, maintained at the Poultry Research Centre in Edinburgh. These birds were injected at one day old with 0.2 mls. of blood from adult birds showing clinically distinguishable signs of osteopetrosis, namely, thickened bones. Only birds which showed thickened bones were used as sources of material for this

initial survey, and birds which were injected, but did not develop osteopetrosis, were not examined.

RESULTS AND DISCUSSION

This survey was mainly directed towards determining whether particles of the expected type were associated with the diseased tissues. A brief account of the electron microscopy picture of the cells is also given. There are several good accounts in the literature of the spectrum of hyperplastic and neoplastic lesions associated with osteopetrosis, which have been examined histologically with the light microscope (Pugh, 1927; Jungherr and Landauer, 1938; Holmes, 1958; and, more recently, Campbell et al, 1964). Because of this, only the intimate cell-particle relationships will be described, rather than extensive electron microscope histological details. In this way, it is hoped that the involvement of the virus will be made more apparent.

No virus-like particles were found in any of the tissues from normal birds which had not been inoculated with virus preparations.

Periosteum. This layer of dividing cells was obviously very much thickened in florid cases of osteopetrosis, but the basic cellular components appeared to be similar to those of normal periosteum. The electron microscope revealed large numbers of osteoblasts, which, although normal in most respects, showed some characteristic differences from the normal cells. The endoplasmic reticulum appeared to be much less developed than that of normal osteoblasts, and, in general, there was

a marked reduction in the degree of differentiation of the cytoplasm. The nuclei of osteopetrotic periosteal cells differs from those of normal cells, in that they show accumulations of chromatin around the edges, just inside the nuclear membranes. Infected nuclei also appear less regular in their morphology, and the cell membrane often appears indented. The peripheral cytoplasm occasionally has small electron dense structures, close to the cell membrane, which are not seen in the normal cells. The intracellular matrix appears to have a great deal of collagen, but the mineralisation in the underlying bone seems to be much less extensive than in normal bone: the layer of osteoid is much thicker than expected.

Around the cell membranes of the periosteal cells clusters of small round objects, with dense centres, were found. These particles were approximately 1,100Å to 1,200Å in diameter, were invariably extra-cellular, and were never found inside these cells.

Plate 1, page 34, shows a typical cell from the periosteum of a florid case of osteopetrosis. The cell appears to be relatively normal, although the sparse endoplasmic reticulum and lack of cytoplasmic detail are obvious. The unusual chromatin distribution can also be seen. The most interesting feature of this plate is the number of particles situated along the cell membrane.

The distribution of the particles was quite extensive in birds with enlarged bones, and most cells appeared to be

associated with them, although the numbers per cell varied a great deal. The intimate associations of the particles with the cell membranes are shown in plates 9,10,11,12. In these plates, various different 'degrees' of association can be clearly seen, but, even so, all the particles are still extracellular.

Examination of the bone underlying infected periosteum revealed that the number of particles associated with the osteoblasts grew progressively fewer, the further towards the centre of the bone the specimens were taken from. The same characteristics were exhibited by the periosteal osteoblasts, shown by those found in the bone. The general pattern of bone ossification appeared to be like that of normal bone, but with a rather more random nature of 'layering'.

Muscle Lesions. Although several different cell types were recognised in the muscle lesions, these lesions seemed to be composed mostly of degenerate or abnormally differentiated muscle cells. These cells were easy to distinguish, since they contained the easily recognisable fragments of muscle myofibrils. Other cells which were frequently seen, were plasma cells, fibrocytes and multinucleated cells resembling myoblasts. With the exception of the blood cells, the muscle cell-types gave the impression of being related in a degenerative, or an abnormally proliferating, sequence. Both the muscle cell, and the connective tissue type of cell, showed clusters of viruses in intimate contact with the cell membranes. In this location, the regions of cell membrane associated with the particles were somewhat

less extensive than those found in the periosteum. However, as can be seen from plate 2, page 35, there are numerous 1,100Å to 1,200Å particles associated with lesions. The periphery of the lesions often merged indistinctly with normal muscle tissue. Here, the difficulty in distinguishing degenerate cells from abnormally differentiating stem cells, is apparent. One cell contains highly differentiated muscle myofibrils, another only a few traces of fibrillar remnants.

Kidney. The kidneys of florid cases of osteopetrosis appeared to be extensively infected with particles similar in structure to those found in the periosteum and muscle lesions. Several different regions of the kidney appeared to have high concentrations of particles, although in the majority of cases examined, the cells in association with these particles did not seem to be obviously different from the normal kidney cells examined. Considerable quantities of particles were found in the glomerular region, on the epithelial cell side of the blood/urine barrier. Particles were not found in the blood in the region of the glomerular tuft, but large numbers were frequently seen in the tubule (the urinary space) around the epithelial cells which extend over the capillaries (plate 3, page 36). These cells often have bud-like processes terminating in electron dense tips, which are structures never observed in the normal kidney, (plates 10, 11, 12, pages 43, 44, 45). It is obvious that very high concentrations of the particles can build up in this region of the kidney.

Further down the nephron, the particles, whilst still found

in the lumen of the tubule, are also found in other locations. At the junction of adjacent cells, and between kidney cells and the basement membrane surrounding the tubules, small concentrations of particles are found. In these regions, individual particles are often observed. Perhaps it is of interest, at this juncture, to point out the ease in distinguishing distal and proximal sections of the tubules: the designation of the cells is readily made. Particles have been seen in both the distal and proximal sections of the tubules, and there appeared to be no preferential association. However, particles were not found in close contact with the adjacent surfaces of the cells lining the collecting tubules, although many virus particles were often found in the lumen at this point. A more complete picture of kidney involvement will be mentioned later in Section V of this work, but the observations reported above seemed to be the most commonly found pattern of distribution of the particles.

It was assumed that, since there was a high concentration of particles in the periosteum, muscle lesions and kidney, and since it has been previously shown that the blood was infective, there would be a high concentration in the reticulo-endothelial system. The liver and spleen were next examined.

Spleen. This is an extremely difficult organ to study, since the organ's ultrastructural morphology has not been completely described in detail (Rhodin, 1963b). Only identification of 'some' individual cells was made, and the general pattern of distribution of the particles in the spleen is obscure. It is probable that there is an association between the

reticular cells and the particles, but, since the identification of cell types is on the basis of eliminating the more easily recognisable groups, then this assumption must be tentative. However, the particles were frequently found associated with collagen fibres (plate 4, page 37). In this organ, particles were found in intra-cellular vacuoles for the first time. The cells in which the particles were found were classified as macrophages, and this was the only tissue, together with the blood, in which intra-cellular particles were observed. The concentrations of particles in the spleen were usually very sparse, and even in florid cases of osteopetrosis, where large numbers were found in the kidney, periosteum and muscle lesions, the groups consisted of only a few particles.

Liver. Kupffer cells in the liver are well known for the rate at which they take up free particles from the blood. However, until now, only very occasional particles have been found in intra-cellular spaces. Particles have never been observed between cells, or in contact with cell membranes.

Testes. The testes have been previously described as juvenile, and contained large numbers of closely packed spermatogonia and spermatocytes. There appeared to be no lumen in the seminiferous tubules, and consequently, most of the typical features of the mature testes were missing. The interesting discovery here was that, even around these undifferentiated spermatogonia precursors, a small, but relatively constant, number of particles were observed (plates 5, 6, pages 38, 39).

Ovary. The picture of the ovary was comparatively similar

to the testes, in that the organ was composed of juvenile, closely packed, cells. In this organ, the cells were mostly germinal epithelial cells, closely packed, but oocytes were not examined in detail. However, particles were found at about the same concentration as that found in the testes, (plates 7, 8, pages 40, 41).

Pancreas. This organ appears essentially normal in most infected birds. A large proportion of the tissue is composed of pyramidal acinar cells forming lobules, which are bound together by loose connective tissue, rich in capillaries. Consequently, since the normal and infected organs appeared to have identical morphological features - even at the ultrastructural level - it was with some surprise that once again particles were observed around the peripheries of the cells. The only obvious macroscopic difference between the infected pancreas, and pancreas from control birds, was the presence of small black spots on the surface of the infected organ. These spots had the appearance of melanotic granules but the complete electron microscope description was not attempted, due to the difficulty which was encountered in the isolation.

Marrow. The marrow of infected birds, as suggested by light microscope work, was basically normal. The tissue contained rather more blast-type cells than would normally be encountered, but there was no leukaemia or any abnormal cells. The large numbers of young, undifferentiated cells, was probably due to the constriction of the marrow cavity by the abnormally proliferating bone. A few particles were observed in vacuoles

contained in macrophageous blood elements.

CONCLUSIONS FROM THIS WORK

The morphological changes from the normal, associated with this pathological condition, have been dealt with only briefly, since the object of this work is to characterise any transmissible agent found responsible for osteopetrosis.

Obviously, the first conclusion which can be drawn from this electron microscope survey is that a particle, approximately 1,000Å in diameter, is associated with osteopetrosis. A particle of this type is possibly the causative agent for the bone and muscle lesions, since these lesions contain very large numbers of particles. Evidence from the filtration experiments add further proof of the relationship of a particle of this size with osteopetrosis. However, this evidence does not in any way prove the causal relationship of particle and pathological condition. Firstly, the particle must be characterised.

The results of this examination suggest that the particle is, in fact, a virus, for the following reasons:

1. the general morphology of the particle is similar to that of other ether sensitive, myxoviruses of the Rous, or leukaemic, type:

2. tissues in which high concentrations of particles were found, exhibited the phenomenon of budding from the cell membranes. This feature is a characteristic of the previously mentioned Rous type of virus, and the sequence of budding described in more detail in plates 9, 10, 11, 12, on pages 42, 43, 44, 45, is typical of this group of viruses. An interesting

feature of this budding which does not appear to have been reported previously for any other similar virus, is the localisation in the cytoplasm of small densely staining osmiophilc granules near the cell membrane of infected cells. These particles are probably ribosomes, but appear to be in larger numbers in the cytoplasm adjacent to cell membranes which are actively budding off viruses. Further study of these structures could prove very interesting, if they are, in fact, related to the replication cycle of the virus:

3. Following from the assumption that these particles are being produced at the membrane of cells, the presence of intracellular virus is probably due to phagocytosis. Only cells like the macrophages of the blood have intra-cellular virus, and these cells do not show budding. It is then probable that normal phagocytosis is not the normal method of entry, by the virus, into the cell.

4. Holmes (1958) claims that the route of vertical transmission from parent to offspring is via the egg (either with the ovum or the sperm). This observation, coupled with the fact that virus particles are found in the testes and ovary, (even though at the stage examined, these organs were non-functional), suggests a causal relationship between the 1,100Å particles and osteopetrosis. Contamination may, of course, take place from the urine, which is possibly, on the evidence presented here, the normal route for horizontal transmission.

Having now tentatively established the presence of virus-like particles in the tissues, it was considered important to verify this point beyond reasonable doubt. The following section reports a series of experiments designed to do this.

TABLE 2

CONDITIONS ASSOCIATED WITH OSTEOPETROSIS, REVEALED BY POST MORTEM AND LIGHT MICROSCOPE HISTOLOGICAL EXAMINATION

Type of lesion	Age in months at termination of experiment												Total
	1-2	2+	3+	4+	5+	6+	7+	8+	9+	10+	11+	12	
Bone	2	28	37	23	11	9	1	3	1	0	2	1	118
Muscle	0	0	4	4	3	3	1	0	0	0	0	3	18
Renal cyst ...	0	2	5	3	1	2	1	2	0	0	0	5	21
Renal tumour .	0	0	1	0	2	1	1	0	0	1	1	5	12
Monoblastic tumour	0	0	0	0	0	0	0	0	0	0	0	4	4

Total number of birds injected with whole blood = 232

% developing bone lesions = 51.4

PLATE 1

Periosteal cells from a typically thickened bone of an osteopetrotic bird. An osteoblast can be seen in the centre of this plate with a large number of 1,100Å particles around the periphery of the cell.

The line underneath this, and subsequent, micrographs represents 1μ unless otherwise stated.

PLATE 1

PLATE 2

Muscle lesion cells. The cell in the upper part of the plate has remnants of myofibrils in the cytoplasm. However, the cell in the centre of the plate is probably a fibrocyte. Both cells have 1,100Å particles in invaginations of their cell membranes.

PLATE 2

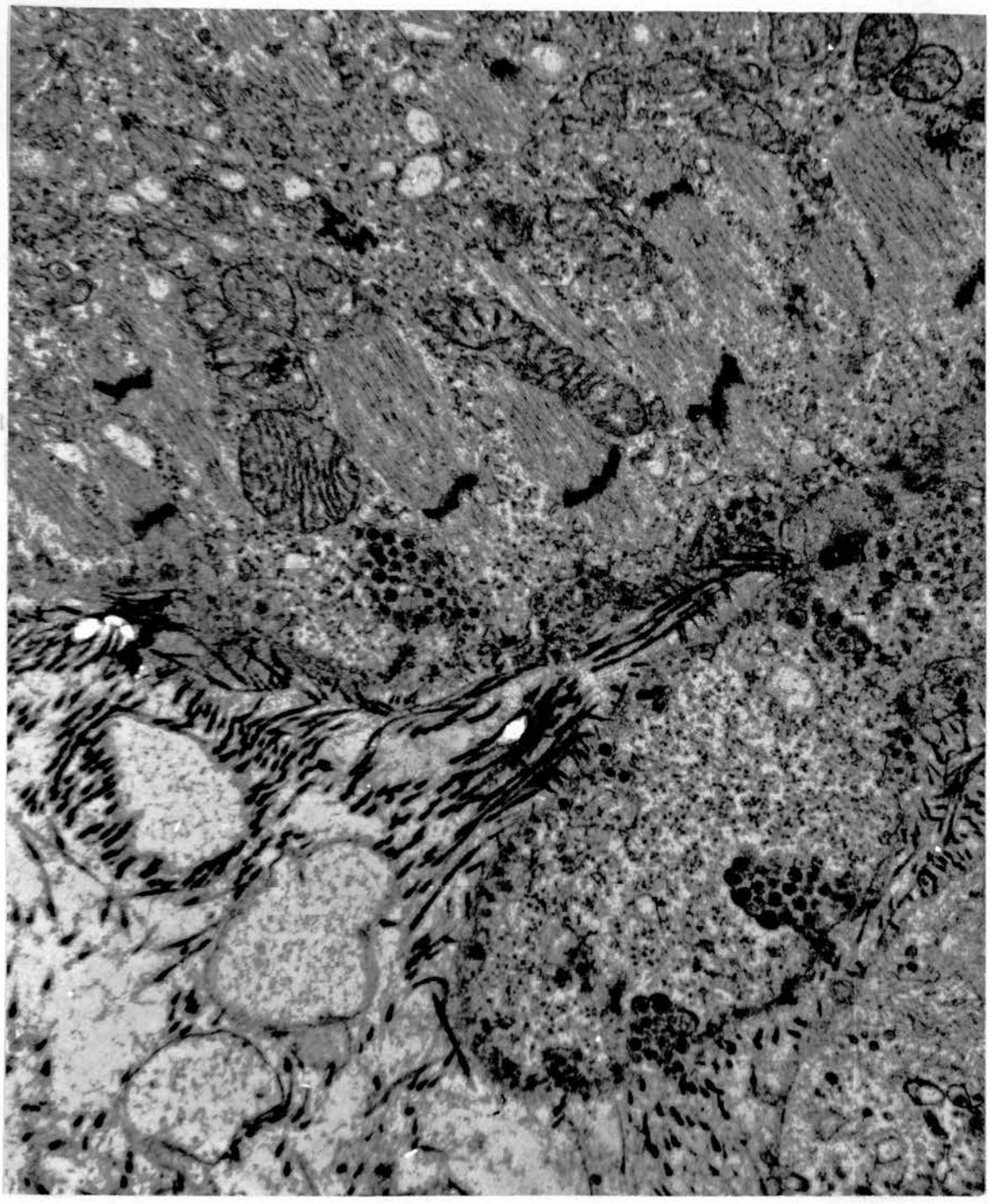


PLATE 3

Glomerular region of a kidney from a bird infected with osteopetrosis. Many particles can be seen in the urinary spaces and occasionally bud-like processes can be seen in the cell membranes of the epithelial cells. No particles are visible in the blood capillaries.

PLATE 3

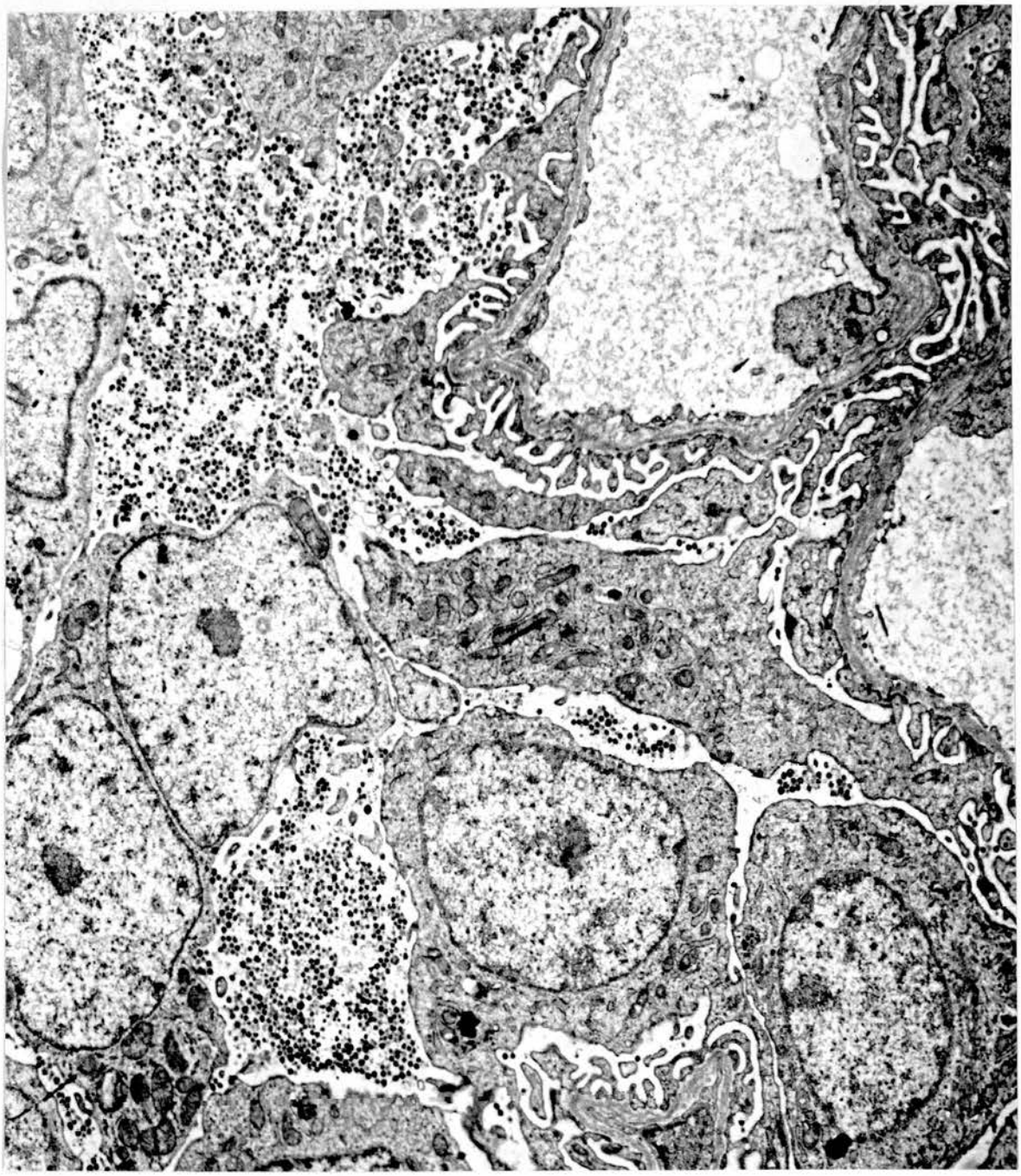


PLATE 4

Spleen from a bird infected with osteopetrosis. These cells are probably reticular cells interspersed with collagen fibres. Several particles can be seen amongst the collagen fibres in the centre of this plate.

PLATE 4

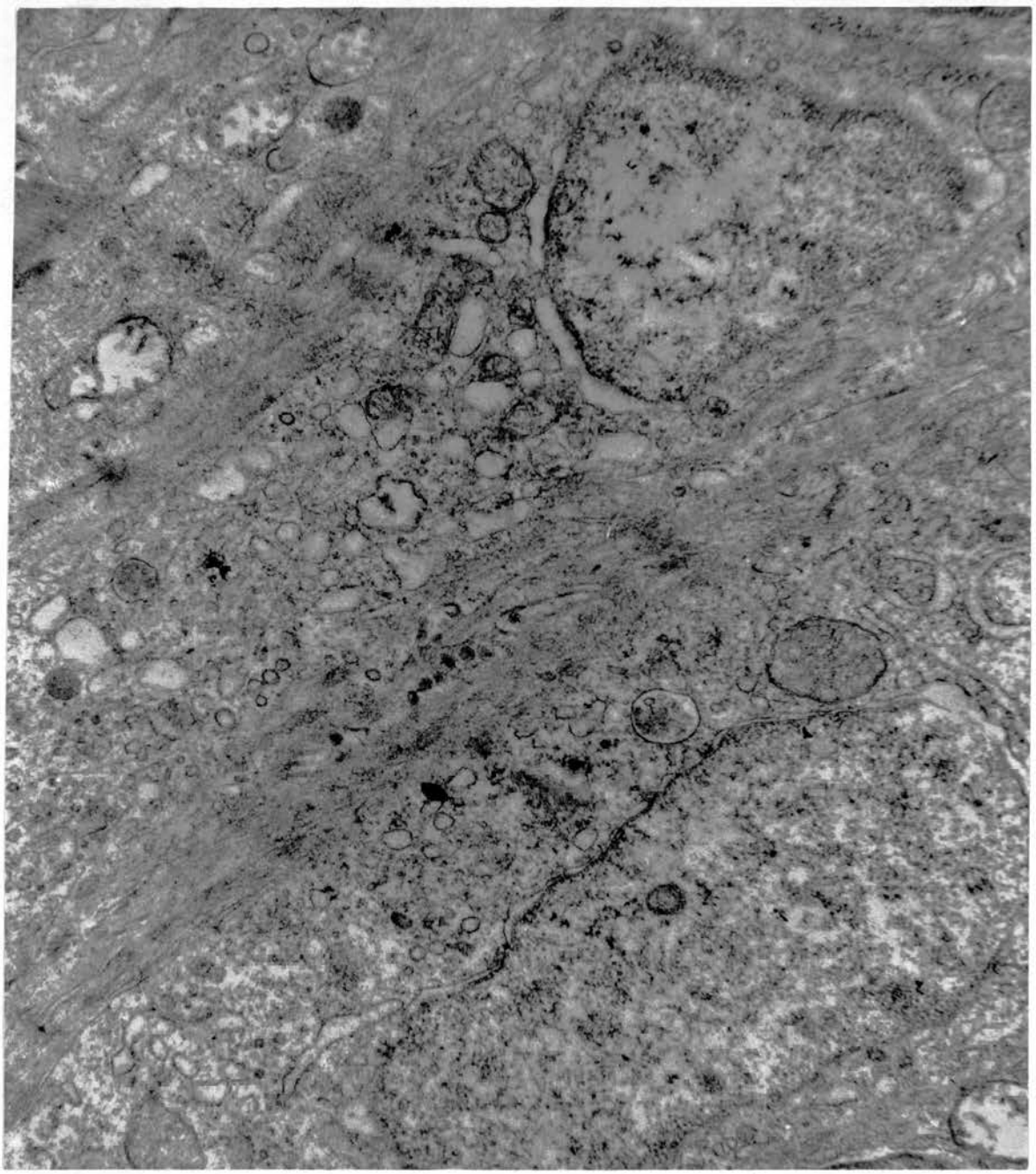


PLATE 5

Testis from a bird infected with osteopetrosis. The seminiferous tubules are completely filled with undifferentiated spermatogonia and spermatozoa. Three single particles can be detected in the intercellular spaces.

PLATE 5

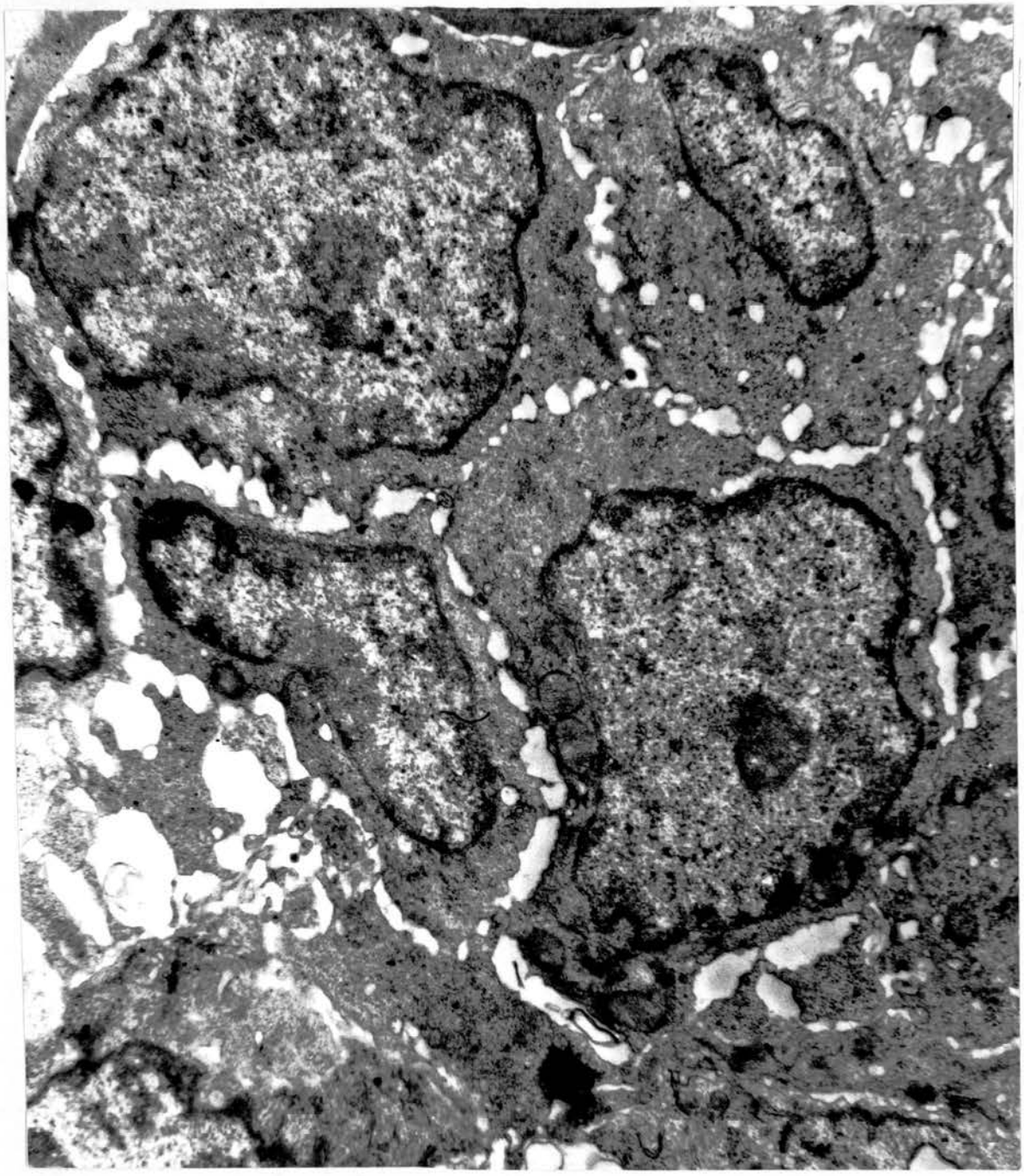


PLATE 6

This is a higher magnification of part of the previous plate. A single particle can be seen between two adjacent spermatocytes.

PLATE 6

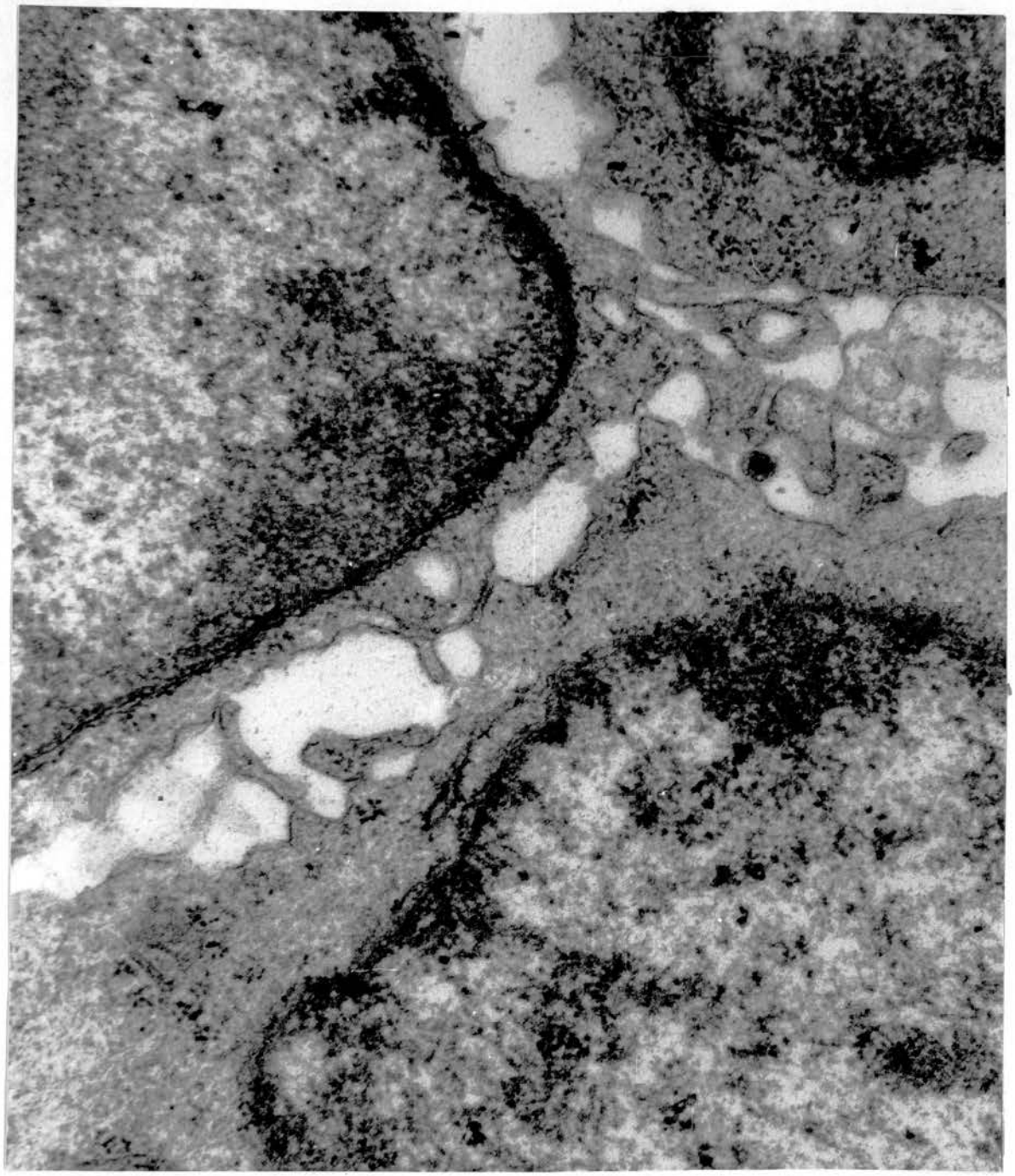


PLATE 7

Low power micrograph of part of an ovary from an osteopetrotic hen. Particles can be seen in the collagenous layer running from the top left to the bottom right.

PLATE 7

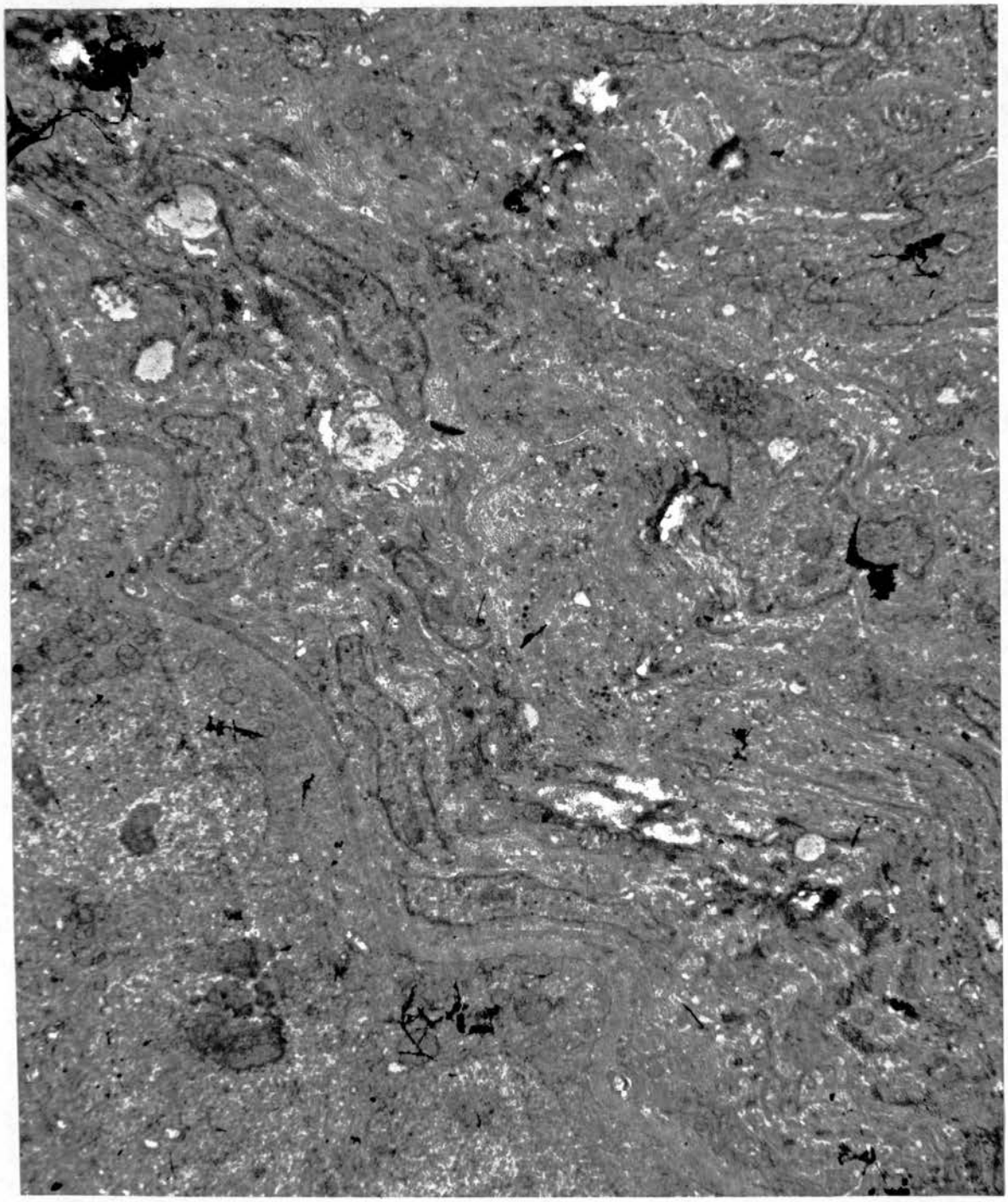


PLATE 8

Higher magnification of part of the previous plate. Particles can be seen amongst the collagen in a similar fashion to those found in the spleen.

PLATE 8

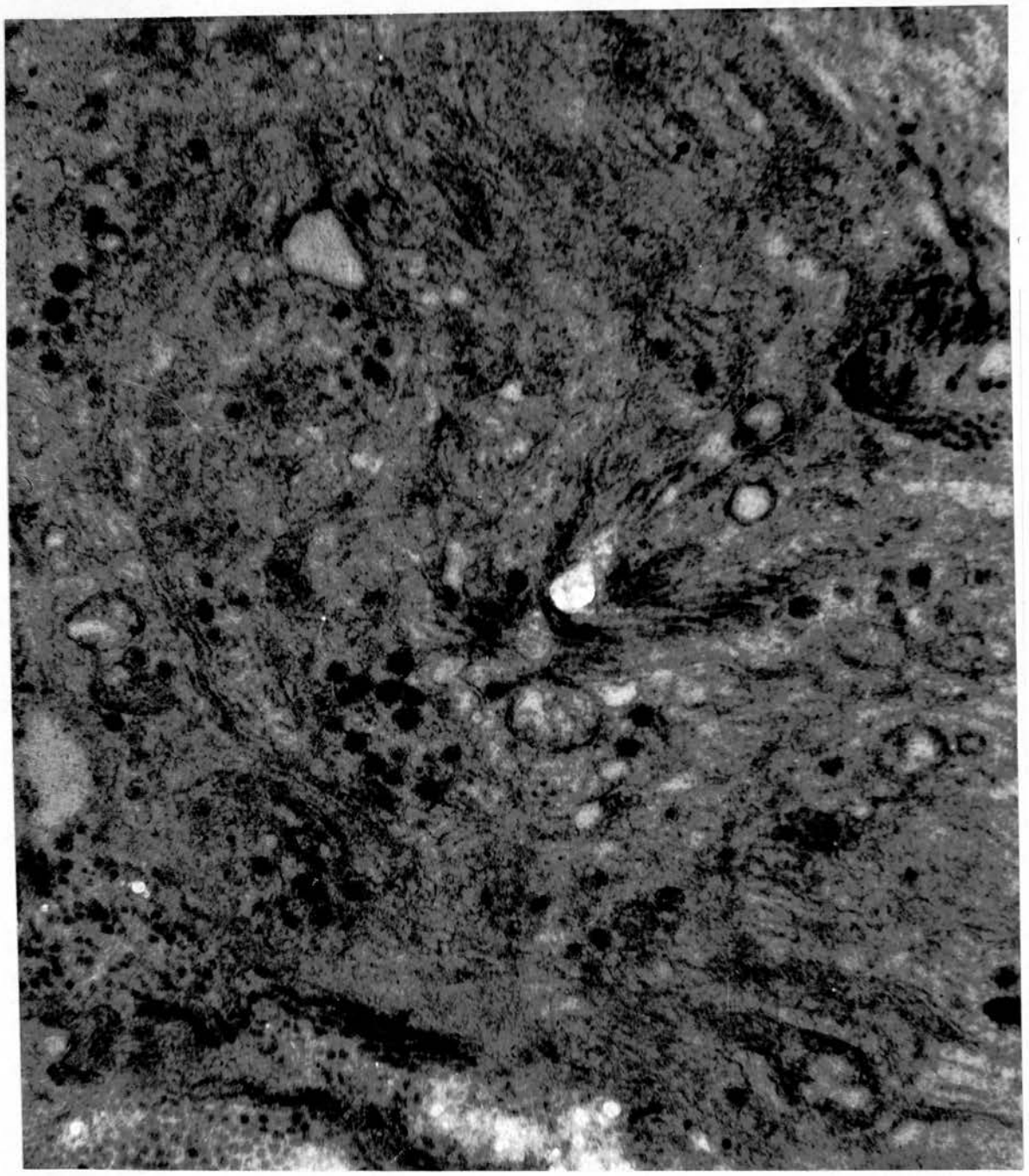


PLATE 9

Part of a periosteal cell. This micrograph shows the intimate association of particles with the cell membrane.

PLATE 9

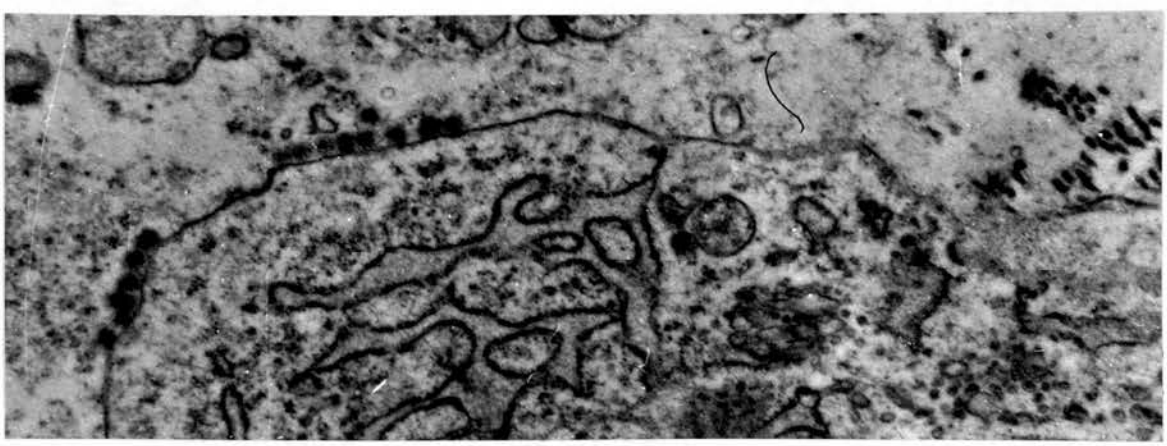


PLATE 10

Kidney epithelial cells: this micrograph shows budding from the cell membrane. A considerable number of ribosomes appear to be concentrated in the cytoplasm near to the buds.

PLATE 10

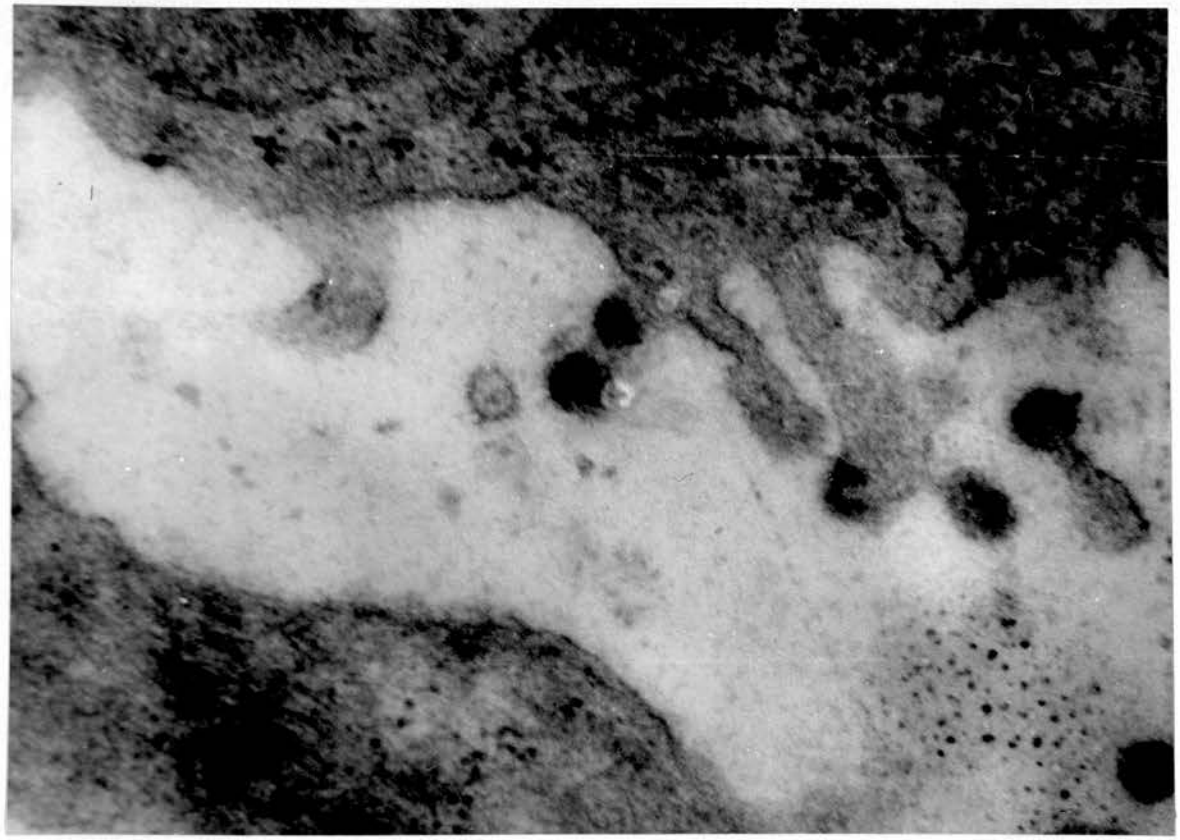


PLATE 11

Kidney epithelial cells showing budding.

PLATE 11

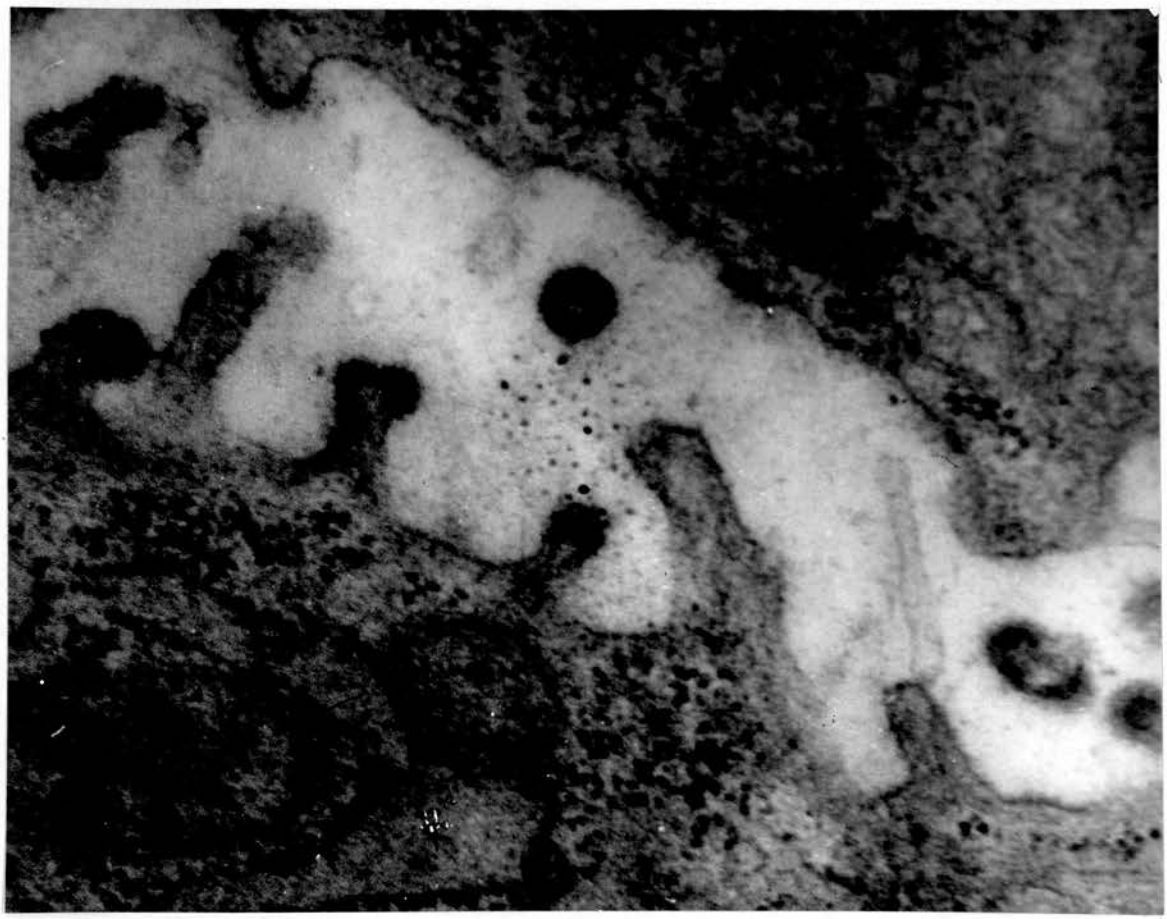
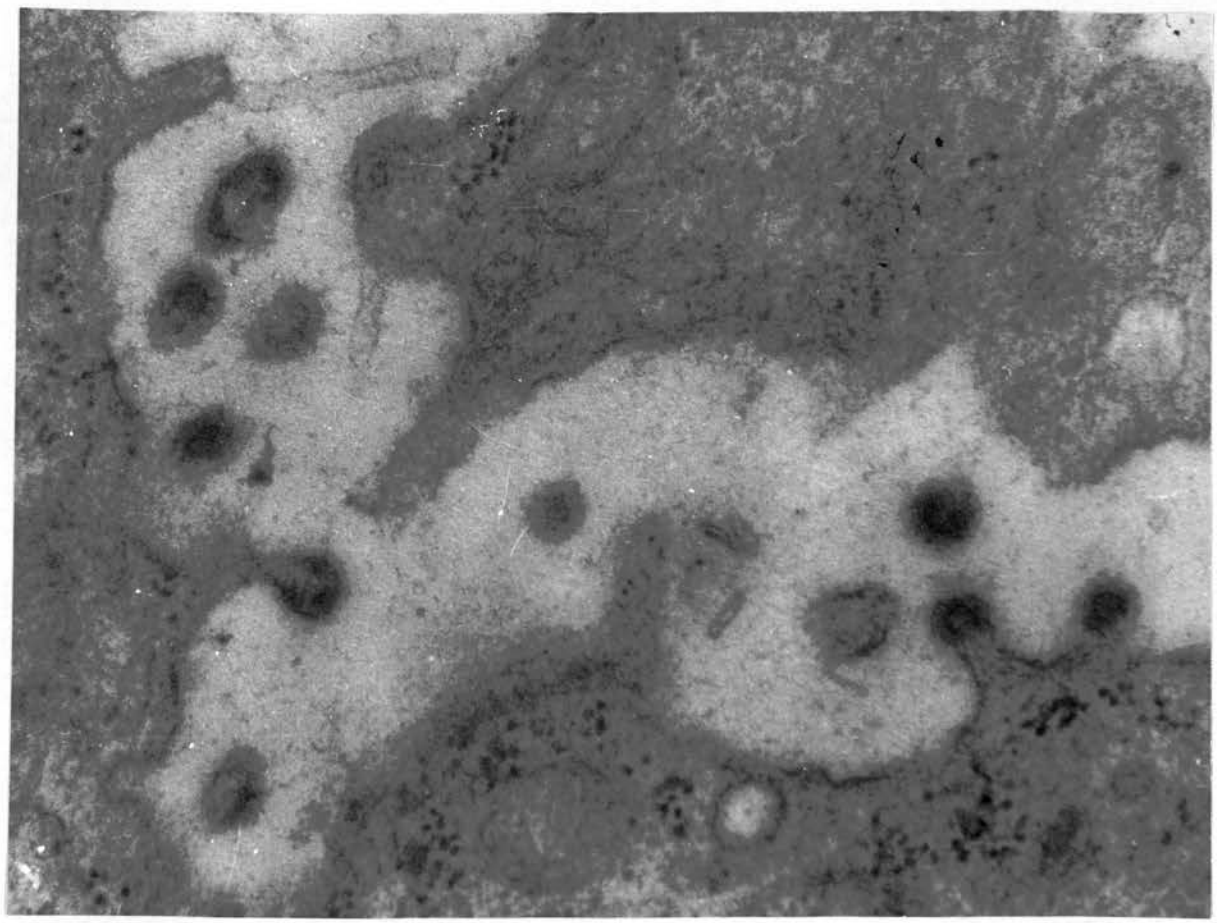


PLATE 12

Kidney epithelial cells showing budding.

PLATE 12



SECTION III

THE CHARACTERISATION OF THE OSTEOPETROSIS VIRUS

INTRODUCTION

In order to prove that the particles observed were viral particles, and indeed the causative agents, a number of tests were devised. The importance of these tests cannot be too strongly emphasised, since it is extremely difficult to demonstrate, conclusively, the causative agent of a virus-induced pathological condition. The dangers inherent in this type of false assumption are so great that workers, particularly in the field of human virology and pathology, should take the greatest care in its establishment. Passenger viruses are ~~not~~ commonplace, and the case of recent observations on tissue cultured material obtained from a case of Birkett's lymphoma serves as an example of the confusion that these 'coincidental' viruses can cause. In this case (Epstein, 1964), tissue culture preparations from the tumour, after having been passaged for eight or nine generations, were shown to contain no fewer than nine different viruses. Some of these viruses were shown to be of known types, and could be classified easily, whereas others could not.

The sort of question which should be asked in the investigation of a condition thought to be virus-induced, where a typical cellular particle is demonstrated, should be "Is this a virus, and, if so, is it a passenger, or the causative agent?" Consequently, the aim of the next part of this work was, firstly, to prove that the particles were indeed viruses, and secondly,

to prove that they were the causative agents.

Several techniques were used to determine these two points, and those used to show the particles to be viruses, on the basis of their similarity with other known viruses, are described below. The different types of tests are here described.

1. Knowing something of the distribution of the particles, from the electron microscope survey, it was considered probable that a light microscope histological examination of infected tissues would reveal considerable information of the particles' composition. It was also thought that histochemical procedures might be adapted for use in the electron microscope. This factor was constantly borne in mind in the choice of all procedures.

2. After examining sectioned material, isolation of the particles was attempted, in order to compare the specificity of sectioned stained material and the isolates. Some purified virus particles have revealed considerable details of their structure by the negatively staining technique used in electron microscopy, (Horne et al, 1959). This technique has also been very useful in assay work, and it was hoped that a quick count of the number of virus particles in a preparation could be carried out in this way, (Watson et al, 1963). Unfortunately, previous reports of this type of treatment of virus particles of similar morphological type have not been favourable. However, this technique of negative staining is useful for checks on the purity of virus preparations.

3. A high resolution examination of the osteopetrosis

particles was carried out in order to compare their characteristics with those of similar known viruses.

4. Finally, two accepted characteristics of viruses, namely ether sensitivity and haemagglutination, were tested. These two properties can help in the classification of a new virus, since both can relate to the outer coat of the particle: ether denatures this coat and renders the particles uninfective, (Andrews and Horstman, 1949), and the haemagglutination test works only if there are haemagglutinating groups associated with the virus, (Anderson, 1959). Haemagglutination is an extremely good assay technique for viruses which react with red cells.

HISTOLOGICAL EXAMINATION OF INFECTED TISSUE

MATERIALS AND METHODS

Light Microscopy. There are many histological techniques available for the specific localisation of cytoplasmic constituents, and only a selected few were used. However, in each case the fixative, stain or extraction technique used in this work, was, for the component under consideration, chosen for its apparent established specificity. A rather detailed account of each method is included here, so that this specificity, or its limitations, can be considered. The standard works of Brachet (1953), Culling (1963), Danielli (1953), Glick (1949), Gomori (1952), Kopac (1955), and Lison (1936) (1953) were used as guides to techniques and procedures.

Tissues. All the tissues examined in Section II of this

thesis were routinely examined with haematoxylin and eosin, and methyl green pyronin. However, since the infected kidney tissue and the muscle lesions showed the biggest concentrations of virus particles, these two tissues were chosen for the lipid, protein, glycogen and detailed nucleic acid determinations.

Fixatives. All the specimens were sectioned after dehydration and embedding in paraffin wax by standard techniques. The enzymic work was carried out after the paraffin had been removed or more frequently in sections cut on a freezing microtome. This latter technique gave extremely good results with glutaraldehyde fixation. On the evidence accumulated from the literature, it would appear that glutaraldehyde is both theoretically and practically the best fixative available for both light and electron microscopy, (Sabatini et al, 1962).

1. Carnoy's Fluids (1 and 2) (Nuclear Fixatives). These gave excellent results with ribonuclease digestive, but unfortunately gave a great deal of cytoplasmic distortion, and generally shrank the tissue. Shrinkage could be reduced if the specimens were kept at $0^{\circ}C$. during fixation. Unfortunately, water tended to remove the basophilia from cells fixed in this way, but, even so, this was a good fixative for determination of nucleic acid distribution.

2. Formal Saline. This is a good general fixative, provided it is buffered to prevent the formation of formal pigment, and provided the tissue is well washed to remove the excess fixative before histochemical treatment. Formal saline was tried for all the stains and found to be the best one for fat tests.

3. Zenker's Formal. Like formal saline, this was used with fairly good results, giving good cytoplasmic fixation. However, although Brachet (1953) recommends Zenker's with R.N.A. digestion, it was found to form insoluble precipitates due to the mercuric chloride. Zenker's was not used for any nucleic acid determinations, but for all the other estimations.

4. Newcomer's Fluid. This fixative penetrates rapidly, and was originally devised for the fixation of chromosomes. The preservation of the chromosomes is better than with Carnoy's fluids, and the Feulgen reaction is consequently much improved, (Culling, 1963).

5. Osmium Tetroxide. Since osmium acid was used extensively for the electron microscope work, it was also tried as a fixative for light microscopy. The penetration was so slow, only pieces two to three millimetres cubed could be treated; these were too small to be practicable for light microscopy.

6. Glutaraldehyde. The buffered solution identical to that used for electron microscope preparations was tried for light microscopy. This proved to be most effective, and will probably become more generally accepted as a light microscopy fixative. Since preservation of morphological detail is adequate for electron microscope work, and the preservation of enzymic activity is also very good, (Sabatini, 1962), this fixative was used extensively.

Serra's solution, Bouin's, Fleming's and Schaudin's solutions were also tried, but were not as effective as the fixatives reported above.

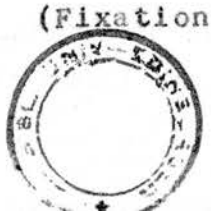
RESULTS AND DISCUSSION

The descriptions of staining techniques, the results, and the discussions for each stain used have been considered together, in order that the conclusions from this part of the work can be appraised more easily.

Haematoxylin and Eosin. (Fixation: all fixatives described above). This is the routine stain which is most commonly used, due to its durability, easy differentiation, and comparative permanency (Culling, 1963). For all routine examinations, Mayer's haemalum was chosen since it gives a good differentiation of general cytoplasmic detail. No details of particles, corresponding to those seen in the electron microscope, were seen in tissues examined with this stain.

Glycogen detection. (Fixation: Formal saline, glutaraldehyde, Newcomer's fluid.) It has been concluded by Hale (1957) and Bensley (1939), in comparisons of the methods for demonstrating glycogen microscopically, that the Bauer Feulgen method (Bauer, 1933), was by far the best technique. This reaction depends on the action of the aldehydic groups of the carbohydrate on the Feulgen reagent. Fresh saliva was used to remove glycogen before staining in control sections. Glycogen was found only in very small, intra-cellular concentrations staining bright red: there was no indication of intercellular concentrations of this stain at the bases of the proximal or distal cells which lined the tubules, or around the cells of the muscle lesions.

Protein Tests. (Fixation: Formal saline, glutaralde-



hyde.) Mazia et al (1953) described the adaptation of bromophenol blue, as used for protein tests in paper chromatography, to cytochemistry. Basic protein will stain without the mercuric chloride, but other proteins bind the dye by coupling through the Hg group. Structures joining basic proteins show enhanced staining after removal of nucleic acids by trichloracetic acid. The stain was tried with and without mercuric chloride. The cytoplasm stained bright blue, and the nucleus a darker blue. In none of the sections examined was the periphery of the infected cells in any way different to that of the non-infected cells.

Lipid Tests. (Fixation: Formal saline, glutaraldehyde.) Formalin fixation has traditionally preceeded staining for fats. Burden (1946) has studied the chemistry of the specificity of sudan black, and has concluded that its specificity is high for neutral fat. However, Chiffelle and Putt's propylene glycol method (modified by Culling, 1963), gave an apparently more definitive stain. In sections treated with these stains, and particularly in the kidney tissue, there was a suggestion of cell membrane thickening, in the regions between the basement membranes of the tubules and the bases of the cells. Osmium tetroxide used as a stain after glutaraldehyde fixation also indicated probable inclusions in these regions. Of the two fat stains, osmium tetroxide, staining after glutaraldehyde fixation, was the best method of showing the lipid localisation. Danielli (1953), however, reports that the mode of action of osmium tetroxide is uncertain, but, even so, he recognises a degree of selectivity of the stain for lipid inclusions.

This lipid test was the first one to indicate the existence of particles in the light microscope. It is not surprising that structures preserved so well in electron microscopy preparations should be visible in the light microscope.

Examination of Nucleic Acid Content of Osteopetrosis infected Kidney and Muscle Lesion Tissue.

Differential extraction techniques. The best methods of detecting nucleic acids are those based on the specific ultraviolet absorption at 2,600Å: these were not available, and so whole cell staining methods were again used throughout. In each stain used, sections of infected and normal tissue were compared with other similar material, after the following treatments.

1. Hot trichloracetic acid (T.C.A.) was used to remove ribonucleic acid (R.N.A.) and desoxyribonucleic acid (D.N.A.), as described by Kurnick (1955), Pollister and Ris (1947).

2. 10% perchloric acid, at 4°C.: this treatment removed R.N.A. differentially, leaving D.N.A. This extraction was used in conjunction with toluidene blue and methyl green pyronin staining, as recommended by Brachet (1957) and Di Stephano (1952).

3. N-HCl at 60°C.: extracts R.N.A. and depolymerises D.N.A.; consequently, this reaction is an integral part of the Feulgen reaction, and can be used for differential nucleic acid tests.

4. Specific digestion with R.N.A.-ase: the lack of specificity, suggested by Danielli (1953), due to contamination by

other enzymes, interference by proteins having access to the nucleic acid substrate, and the inaccessibility of the 'core' of R.N.A., (Loring et al, 1947), are difficulties which have now been overcome. This has been due to the preparation of crystalline enzymes (Kurnitz, 1950), increasing the specificity by improving the purity. The proteolytic enzyme associated with R.N.A.-ase preparations are in such low concentrations that they have no effect on tissues and give no reason to suspect the value of R.N.A. localisation, (Brachet, 1957; Kurnick, 1955). In the treatment of osteopetrosis infected tissues, an apparently non-specific inhibition of the enzyme was encountered. This was found to be caused by the presence of metal ions which had been introduced into enzyme solutions during weighing procedures.

Staining Techniques for Nucleic Acids.

1. May-Grunwald Giemsa. (Fixatives: Carnoy's fluid, glutaraldehyde.) Jacobsen and Webb (1952) reported differential staining of nuclei, nucleoli and basophilic cytoplasm, by using May-Grunwald stain, followed by Giemsa. When the purple-red coloured regions in these sections were compared with the positively staining regions in Feulgen preparations, identical results were recorded. This method was claimed to be more sensitive than methyl green pyronin as a test for R.N.A., since the red R.N.A. pyronin stain can be masked by the methyl green D.N.A. stain. Jacobsen and Webb also showed, by isolation experiments, that desoxyribonucleoprotein (D.N.P.) and ribonucleoprotein (R.N.P.) also took up the stain, D.N.P. staining purple-red, R.N.P. staining blue.

This characteristic was used successfully by ~~Horne (1957)~~ to detect R.N.P. and D.N.P. In the experiments carried out with this stain to determine the presence and nature of the virus particles, there was no suggestion of 'inclusion' bodies in the predicted regions, and there were no detectable differences between infected and non-infected cells.

2. Toluidene Blue. (Fixation: Carnoy's fluids, glutaraldehyde.) This is an extremely easy stain to use, and Michaelis (1947), in a discussion of basic dyes and nucleic acids, states that T.B. combines stoichiometrically with nucleic acids. Lindegren (1951) places more confidence in this method than in methyl green pyronin. Even though Brachet (1953) thinks it is inadequate when there is a lot of R.N.A., and is also unable to differentiate between R.N.A. and D.N.A., it was thought that this stain might prove useful for the initial detection of the particles. Unfortunately, there were no detectable differences between the control material and the tissue known, from the electron microscope examination, to contain particles.

3. Methyl Green Pyronin (Unna Pappenheim). (Fixation: Carnoy's fluids, glutaraldehyde.) Brachet (1952) has used this stain in conjuction with R.N.A.-ase to demonstrate the presence of R.N.A.; this has been referred to by Abolins (1952) as the Brachet-Pappenheim cytochemical method for R.N.A. This proved to be the best way of detecting the viruses with the light microscope. If used to stain polymerised D.N.A. and R.N.A., a mixture of methyl green and pyronin stains the D.N.A.

selectively with the methyl green. In fact, Jacobsen and Webb suggest that methyl green masks pyronin in tissues containing D.N.A. and R.N.A. This has not been found in the present studies, since nucleoli stand out well, but this may have been due to the use of glutaraldehyde as a fixative, which has not previously been described. Alternatively, with depolymerised R.N.A. and D.N.A., pyronin stains selectively, (Kurnick, 1949). This reaction, in the case of methyl green, is due to a stoichiometric reaction between the Feulgen reagent and the polymerised D.N.A. (Kurnick and Mursky, 1949; Kurnick, 1950). However, it was assumed that in sections stained with methyl green pyronin, red staining corresponded to regions with high D.N.A. contents, and green staining indicated R.N.A. This stain revealed extensive, though narrow, regions of green staining, corresponding to the distribution of particles seen in the electron microscope. These R.N.A. staining regions were at the bases of cells, or, to a lesser extent, between the cells, but appeared rather more diffuse than the electron microscope photographs would suggest. This could have been due to fixation and staining, resulting in diffusion of the R.N.A. away from the original location.

4. Fluorescent Microscopy. (Fixation: Carnoy's fluids, glutaraldehyde.) This work was based on techniques described in Armstrong's review (1956), in conjunction with the fluorescent stain acridine orange at p.H. 3.6 - 5.2. The best staining was found to take place at approximately p.H. 4.3 - 4.5. On occasions, very intense flame-red spots were found in regions

where the virus particles were expected to be, but this stain was found to be inconsistent for sectioned material, although suitable for smear preparations of partially purified virus. It could be concluded from this that the acridine orange is probably detecting R.N.A., and that the virus particles do contain R.N.A.

EXAMINATION OF PARTIALLY PURIFIED VIRUS

INTRODUCTION

The previous histochemical examination revealed that the particles were probably composed, at least in part, of R.N.A. and osmiophilic material. Since the staining of the R.N.A. positive material was merely 'in the same region' as that shown to be occupied by particles in the electron microscope, this evidence gave no indication of the exact location of the R.N.A. with respect to the particle. This R.N.A. may have been due to the synthesising components in the cytoplasm, rather than to the particles themselves. Consequently, the particles were isolated from the infected tissues, in order to demonstrate their basiophilic nature, and the kidney was chosen as the most easily obtainable, and prolific, source of virus.

MATERIALS AND METHODS

Bather (1954) reported that a sequence of high - low centrifugation cycles of macerated tissue containing virus eventually gave relatively pure samples of virus. Kidney tissue and blood were treated by a purification scheme, similar to that described by Bather, and details of the

sequences can be found on page XVIII of the Appendix.

The purity of the resultant pellet was checked by examining negatively stained, spray droplet preparations in the electron microscope, and was found to be relatively free of cell debris: this technique is described later, on page 60. The precipitates obtained from the centrifugation cycle were then treated in the same way as Epstein (1960) treated Rous Sarcoma virus (R.S.V.) after fluorocarbon purification. This involved smearing the final preparation, which appeared fairly homogeneous in the electron microscope, onto several microscope slides. The slides were then treated with either hot T.C.A., 10% perchloric at 4°C., N-HCl at 60°C., R.N.A.-ase digestion, or distilled water, in exactly the same way as the sectioned material in the preceeding section.

All the slides were then stained with acridine orange, according to Armstrong's description, and compared with smears obtained from pellets of normal kidney macerates, prepared in the same way.

RESULTS AND DISCUSSION

When staining R.N.A., acridine orange fluoresces with an intense flame-red colour, as previously described. This is unmistakable, and so the slides were scored arbitrarily as being:-

\+ meaning intense homogeneous red

\- meaning no flame colour at all

± very few, very small specks of red.

Fluorescence. The results can be summarised in the following table.

Treatment	Smears from pellets of normal tissue	Smears from pellets of osteopetrosis infected tissue
T.C.A.	-	-
Perchloric	-	-
Hot N-HCl	-	-
R.N.A.-ase	-	-
Cold water	±	+
Hot water	±	+
Untreated	±	+

In all the slides treated to remove either total nucleic acid or R.N.A. alone, no fluorescence of the flame-red colour could be found. Conversely, all three smears of the osteopetrosis infected tissues, stained after treatment with hot or cold water showed considerable amounts of the flame-red colour. The distribution of the stain in the smears from pellets from infected tissues was such that the whole slide was red, and the colour varied in its intensity only at points at which the smear was too thick to allow the ultra-violet light to penetrate. In smears obtained from normal tissues, there was only very slight fluorescence, in the form of small discrete specks. This could be explained by the fact that on examination in the electron microscope, these control preparations were shown to contain some cellular debris. It was assumed that some of this material was basophilic - being either fragments of endoplasmic reticulum, or even, occasionally, free ribosomes. Consequently,

these contaminants would also take up the stain.

As a result of this examination, it was concluded that, since the smears from infected tissues consisted of relatively homogeneous preparations of virus, and since these preparations gave a much more positive acridine orange reaction, the particle was composed, in part, of R.N.A.

NEGATIVE STAINING PREPARATIONS

INTRODUCTION

The examination of viruses by this technique has been frequently used for the investigation of virus ultrastructure. General reviews of the subject can be obtained from the work of Brenner and Horne (1959), Wildy et al (1960), and Kay (1961). Excellent micrographs have been obtained of some viruses, for example, herpes simplex, polyoma-types, rheo-viruses, and the phage group, giving very high resolution of virus detail. Unfortunately, the leucosis virus type, which osteopetrosis virus particles resemble, has proved unrewarding as a subject for negative staining treatment. It appears that, as this type of particle dries on the grid, a considerable amount of structural distortion occurs, with the result that the regular outline is lost. In fact, not only is the original morphology changed, but 'regular' artefacts are encountered. These artefacts are the well known tailed forms of this type of R.N.A. virus, and care should always be taken when trying to explain this type of structure (Dalton et al, 1964). However, since the electron microscope can demonstrate the presence of some virus particles

in concentrated suspensions, it was hoped that a quick counting technique of the type already described, using latex particles, could be developed. This type of quick assay would be extremely useful for the osteopetrosis particles, since growth is so slow in chickens.

MATERIAL AND METHODS

The virus suspensions were obtained by the modified Bather technique, as described in the Appendix, on page XVIII.

The pellets suspended in a minimal volume of ammonium acetate buffer were then mixed (50:50) with a 4% phosphotungstic acid solution (P.T.A.), and sprayed onto grids filmed with formvar and carbon with a Vaponefrin spray gun. In some cases, the P.T.A. solution contained latex particles at a concentration of approximately 10^{10}/ml.: the latex particles were either 880Å or 2,000Å in diameter.

In order to improve the negative staining properties of these particles, some were treated with an Ultrasonicator, in order partially to disrupt them, and improve the penetration of the stain. It was hoped that the ultrasonic degredation would strip off the outer coat of the particles, revealing details of the ultrastructure of the core.

RESULTS AND DISCUSSION

All the preparations of partially purified osteopetrosis virus which were negatively stained proved disappointing, since the virus particles showed no regular outline or surface details, plates 13, 14, pages 75, 76. These observations agree with those of other workers who have examined other Rous-type

particles in the same way. Particles which have approximately the same size as that expected of the osteopetrosis virus have been seen in large numbers, but there has been little consistency in the general morphology. Consequently, although it was shown that there was a relative uniformity of particulate material within the size range expected, these particles could not be definitely recognised as the same as the ones seen in sections. Furthermore, since there were often particles in the preparations whose identities were doubtful, this technique could not be used for counting particles of this type.

The preparations of ultrasonicated material showed particles which were slightly smaller and more irregular in outline than those seen in normal preparations. However, no distinguishing features were seen, which might suggest that the cores had been stripped of their surrounding membranes.

ELECTRON MICROSCOPE HISTOCHEMISTRY

INTRODUCTION

Only recently have methods been evolved enabling electron microscopists to study the localisation of specific materials in biological tissues at high magnification. Good reviews of the subject have been written by Sabatini et al (1962) and Luft (1959). Sabatini's work deserves particular attention, since this consists of an analysis of a large number of possible fixatives, in order to determine which ones are best qualified to satisfy the requirements of both the electron microscopist and histochemist. It was this analysis of

aldehyde fixatives, which fix by the relatively mild action of forming electrostatic bonds, that has resulted in gluaraldehyde being chosen as the most suitable fixative for electron microscope histochemical analysis. Enzyme extraction techniques developed for large pieces of tissue, or for embedded and sectioned material, would not be completely suitable for the study of the fine structure of virus particles. Consequently, a technique was evolved which would satisfy the requirements of specific enzyme extraction and good preservation. It was hoped to determine the component of the osteopetrosis virus particle which was composed of R.N.A.

MATERIALS AND METHODS

Small pieces of tissue (2 - 3 mm^3) were fixed in cold glutaraldehyde, for a period of half an hour. This tissue was frozen and sectioned with a freezing microtome, and then sections were replaced in the glutaraldehyde for another five minutes, to ensure complete fixation. These sections were then thoroughly washed in several changes of distilled water, to remove all traces of the fixative and any enzyme inhibiting contaminants, such as free metal ions. The sections were then ready for the enzyme analysis, which consisted fundamentally of the same sequence of treatments as that used for the light microscope sections.

All the manipulations described here were carried out in small, 0.5 x 5 cms. test tubes, using Pasteur pipettes to transfer solutions.

The sections were:

1. fixed in a 5% glutaraldehyde solution for five minutes:

2. washed with three changes of distilled water:

3. treated with a 0.01% solution of R.N.A.-ase in glass distilled water, and incubated for one hour, at 37°C.:

4. washed in distilled water:

5. fixed in Caulfield's buffered osmium tetroxide fixative for one hour:

6. dehydrated and embedded by the usual methods (page XV of the Appendix) for electron microscope examination.

The advantages of this technique are:

1. the tissue is quickly and completely fixed by a chemically mild process:

2. frozen sections are ideal for the complete penetration of the enzyme:

3. the thin sections enable the fixative to be completely removed by distilled water, so that the possibility of inhibition of the enzyme is eliminated:

4. post-fixation with osmium tetroxide after enzyme treatments results in the substrate not being inactivated before enzyme treatment:

5. subsequent dehydration and embedding procedures do not extract or displace the R.N.A.

RESULTS AND DISCUSSION

The appearance of material treated with R.N.A.-ase in this way showed only slightly inferior preservation to that treated by conventional methods (plate 15, page 77). The ribosomes of

the endoplasmic reticulum system were obviously affected by R.N.A.-ase treatment, as were the nucleoli and free ribosomes. All the particles and structures with a known R.N.A. content, which had been treated with R.N.A.-ase, had a definitely less dense composition that those of the controls: this was a useful guide when examining the virus particles. Particles which had been treated with R.N.A.-ase had a less dense core than those of the controls. The outer membrane system did not appear to be altered by this R.N.A. extraction procedure, which suggests that the virus has an R.N.A. core.

HIGH MAGNIFICATION EXAMINATION OF THE VIRUS PARTICLES

INTRODUCTION

In order to compare micrographs obtained using the Phillips E.M. 100, a few micrographs were taken using an A.E.I. E.M. 6 machine. The resolution of the E.M. 6 is claimed to be better than 8Å, compared with about 25Å claimed for the E.M. 100.

MATERIALS AND METHODS

Specimens examined at high magnification were treated very carefully. Tissues were fixed and embedded by the usual techniques, but cut so that grey sections, daimed by Peachey (1958) to be 600Å thick, were obtained: normally, silver (600 - 900Å) sections were used for routine work. The very thin sections were then mounted on New 400 mesh copper grids, previously cleaned in chloroform, in order to remove any traces of grease. The sections were mounted directly on fine

mesh grids, in order to decrease the adverse effect on resolution, caused by supporting films. Staining was carried out using lead citrate and uranyl acetate. In this examination, all aqueous solutions used in conjunction with the lead stain were made CO_2 free, by boiling the water used, and keeping them in containers provided with soda-lime carbon dioxide traps. This precaution greatly reduced the amount of non-specific precipitation of lead carbonate (and other lead salts). The uranyl acetate was always freshly prepared, centrifuged immediately before use, and sections stained in a petri dish away from direct sunlight, which further reduced non-specific precipitation.

RESULTS AND DISCUSSION

The results of this work are shown in plates 16, 17, pages 79 , and a drawing of the probable structure, based on these micrographs, is included on page 78 , diagram 1.

The particle has an osmiophilic, dense central nucleoid, which is probably composed of R.N.A., as demonstrated in the previous work. This central core is elliptical in shape, and the approximate dimensions are 700Å along the major axis, and 550Å across the minor one: these figures are the means of measurements of 200 different particles. The nucleoids are surrounded by two distinct membranes, giving an overall elliptical shape, of about 1,100Å across the major axis, and 950Å across the minor one. The two membranes surrounding the nucleoid are probably derived from the cell membrane, from which the viruses appear to be, in part, formed, (plates 9,

10, 11, 12, pages 42, 43, 44, 45). The thickness of the virus membranes (approximately 100Å) compares favourably with the accepted thickness of normal cell membranes.

The elliptical shape of the particles is such that a wrong interpretation of the structure seen in the electron microscope can easily be made. An example of how this can be done is shown in diagram II, page 78. The different appearances of sections of the same particle could explain the occurrence of variations in size and morphology of this type of virus particle. Diagram II, page 78, shows how sections of a virus particle, cut in different planes, could vary in their appearance. The virus particle in diagram II has been distorted to exaggerate this effect.

Since particles of this type have also been shown to vary in size and shape due to their degree of hydration, (Bernhard, 1958; Sharp and Beard, 1954), it is difficult to suggest definite dimensions for the osteopetrosis virus particle.

HAEMAGGLUTINATION

INTRODUCTION

A quick and simple assay of certain viruses can be undertaken by determining the ability of serial dilutions of the virus suspension to agglutinate erythrocytes. The haemagglutinating (H.A.) viruses have been divided into three groups, (Anderson, 1959).

1. The myxogroup, in which the haemagglutinating agent is the virus particle itself, but which also has an enzyme (neuraminidase) which allows elution of the virus.

2. A group, which includes encephalomyocarditis and pneumonia virus of mice, which is like group 1, but differs in that the virus does not possess an eluting agent.

3. A group of viruses producing haemagglutinin at some stage in their life cycle, but not directly associated with the virus particle. The basis of the quantitative assaying of viruses using erythrocytes has been reported by Hirst (1942a,b), and the probable mechanism is now generally accepted.

MATERIALS AND METHODS

Red blood cells (R.B.C.s) were sedimented from citrated chicken or sheep blood by centrifugation at 2,000 r.p.m. for five minutes. After three washes, by resuspending and sedimenting in 0.9% saline (chicken R.B.C.s) or P.B.S. (for the sheep cells), a 1% suspension of the packed R.B.C.s was prepared in the appropriate solution. The cells, in suitably diluted samples, were then counted in a haemocytometer. Titrations were carried out in haemagglutination trays which were filled with 0.2 ml. aliquots of the 1% chicken R.B.C. preparations. In each experiment, 0.2 mls. of the undiluted virus suspension, prepared by differential centrifugation from kidney tissue or blood, was placed in the first cup of the tray. Doubling dilutions were made by transferring half of the red cell/virus mixture from the first cup to the second, and then half of that mixture to the third, and so on. The trays were then left at $4^{O}C$ overnight, and examined after about twelve hours.

RESULTS AND DISCUSSION

The haemagglutinating capacity of osteopetrosis virus particles proved to be completely negative. In none of the preparations was there any sign of agglutination. All of the other Rous-type viruses give negative haemagglutination reactions, which agrees with the previous observations that only the Rous-like particles could be seen in the osteopetrosis affected tissues. If other viruses of the haemagglutinating type had been present, obviously an H.A. reaction would have taken place. Consequently, although this negative result is of little value for further investigation of the osteopetrosis virus, it does indicate the probable homogeneity of the preparations, at least with respect to haemagglutinating viruses.

ETHER SENSITIVITY OF PARTIALLY PURIFIED OSTEOPETROSIS VIRUS PREPARATIONS

INTRODUCTION

The inactivation of viruses by organic solvents and bile salts has often been used as a means of classifying viruses. Burnet and Lush (1940) graded a number of viruses according to their susceptibility to sodium desoxycholate and sodium lauryl sulphate and showed the susceptibility to be the same for the two salts.

Andrews and Horstmann (1949) studied the susceptibility of twenty-five viruses to diethyl ether, and showed that crude filtrates of R.S.V. I, which were active to a dilution of 1:1,000, were inactivated to such an extent that only the

undiluted filtrates were infective. They drew attention, not only to the close correlation between the sensitivity of viruses to ether and also bile salts, but also to the fact that viruses like R.S.V. I, which are sensitive to these reagents, are also extremely labile at ordinary temperatures. This sensitivity has been shown to be due to the solubility of the outer lipoprotein-membranes in the organic solvents, (Drayton, 1960a,b). The experiments described below are designed to show the decrease in infectivity of the osteopetrosis virus resulting from organic solvent treatment.

MATERIALS AND METHODS

Partially purified virus preparations from infected kidney tissues, as previously described, were used for this work. Equal volumes of the virus preparations, in 0.9% saline, were diluted with the same volumes of ethyl alcohol, diethyl ether, chloroform and ethylene glycol. These mixtures were shaken vigorously for five minutes, and then centrifuged for one hour at $0^{o}C$. in an angled head centrifuge, at 15,000g. The supernatant was discarded and the precipitate resuspended in 0.9% saline. 0.2 aliquots of these preparations were then injected into day-old chicks: 0.2 ml. aliquots of untreated, partially purified, virus were also injected intramuscularly into other day-old chicks, as controls.

RESULTS AND DISCUSSION

Without exception, each of the organic preparations completely inactivated the partially purified virus. It is now

well known that R.S.V.-type virus consists of an R.N.A. core with two phospholipid surrounding membranes. Consequently, since the morphology of the particles, as shown in the electron microscope photographs, is identical to R.S.V.-type viruses, the inactivation of these particles is probably due to the essential lipo-protein coat being completely dissolved.

CONCLUSIONS

Several different conclusions can be drawn from sections I and II of this work.

1. In the survey of tissues infected, only one type of particle has been observed, although it was found in most of the tissues examined.

2. This particle is, in part, composed of R.N.A., since the light microscope histochemical examination showed a high degree of basophilia in those regions of infected tissues shown, by the electron microscope, to contain particles. Glycogen and protein staining gave negative results for these regions.

3. Enzymic extraction of the core with R.N.A.-ase showed that the central, dense part of the particle, designated the nucleoid, was composed of R.N.A.

4. The particle has a lipid component as shown with the light microscope by its osmium staining characteristics.

5. The presence of a lipid component is further proved by the ether sensitivity of the partially purified preparations

6. The high magnification photographs indicate a structure

consisting of a dense central nucleoid with two surrounding membranes, which are probably the lipoprotein component of the particle. The overall size is approximately 1,100Å.

7. These virus particles are certainly necessary for the development of osteopetrosis since the filtration of infective preparations with millipore filters indicates that the infectivity was associated with particles between 1,000Å and 2,000Å in diameter. The ether sensitivity further substantiates this point.

8. There was no other evidence to suggest the presence of another structural type of particle, so that, although there is no evidence to suggest that the particles are identical from a genetical or immunological point of view, morphologically, the population was homogeneous.

TABLE 3

SUMMARY OF HISTOCHEMICAL EXAMINATION

Stain	Reference	Fixatives	Extraction	Comments
Sections of infected kidney and muscle lesions				
Haematoxylin and Eosin	Culling (1963)	Glutaraldehyde Carnoy's Fluids Formal saline Zenker's formal Newcomer's fluid Osmium tetroxide	None	None of the fixatives, in combination with this stain showed the presence of the particles observed in the electron microscope
Bauer Feulgen	Bauer (1933)	Newcomer's fluid Formal saline Glutaraldehyde	Fresh saliva	No evidence of particles with these fixatives: .˙. particles not glycogen
Bromophenol Blue	Mazia et al (1953)	Formal saline Glutaraldehyde	None	No evidence of particles with either fixative: .˙. particles not protein ones
Sudan black	Chiffelle Putts (modified by Culling, 1963)	Formal saline Glutaraldehyde	None	No evidence of particles with either fixative: .˙. not lipid in particles
Osmium Tetroxide	Marchi's Method (Modified)	Formal saline Glutaraldehyde	None	Small black inclusion bodies found between kidney cells and around edges of cells of liver: .˙. lipid component associated with particles

TABLE 4

SUMMARY OF HISTOCHEMICAL EXAMINATION

Stain	Reference	Fixatives	Extraction	Comments
Methyl green pyronin	Brachet (1952)	Carnoy's fluids Glutaraldehyde	Hot T.C.A. 10% Perchloric N-HCl at 60°C.	Regions which would be expected to contain particles demonstrated in the E.M. stained red with this stain. All the extracting techniques removed the red staining material: .˙. R.N.A. associated with particles.
May Grunwald Giemsa	Jacobsen and Webb (1947)	Carnoy's fluids Glutaraldehyde	Hot T.C.A. 10% Perchloric N-HCl at 60°C. R.N.A.-ase	No detectable differences between normal tissue and tissue infected with osteopetrosis.
Toluidene Blue	Michaelis (1947)	Carnoy's fluids Glutaraldehyde	Hot T.C.A. 10% Perchloric N-HCl at 60°C. R.N.A.-ase	No detectable differences between normal tissue and tissue infected with osteopetrosis.
Acridine Orange	Armstrong (1956)	Carnoy's fluids Glutaraldehyde	Hot T.C.A. 10% Perchloric N-HCl at 60°C. R.N.A.-ase	Rather inconsistant results: acridine orange found to fluoresce in regions where virus particles expected: .˙. R.N.A. particles.
Nucleic acid determination of smear preparations				
Acridine Orange	Armstrong (1956)	None	Hot T.C.A. 10% Perchloric N-Hcl R.N.A.-ase	In all sections treated to remove total R.N.A., or R.N.A. alone: no fluorescence Unextracted preparations from infected tissues showed considerable fluorescence. .˙. R.N.A. present.

PLATE 13

Negatively stained preparation of partially purified osteopetrosis virus preparation. Tailed forms of the particles can be seen, but it is difficult to distinguish the viruses from pieces of cell debris.

PLATE 13

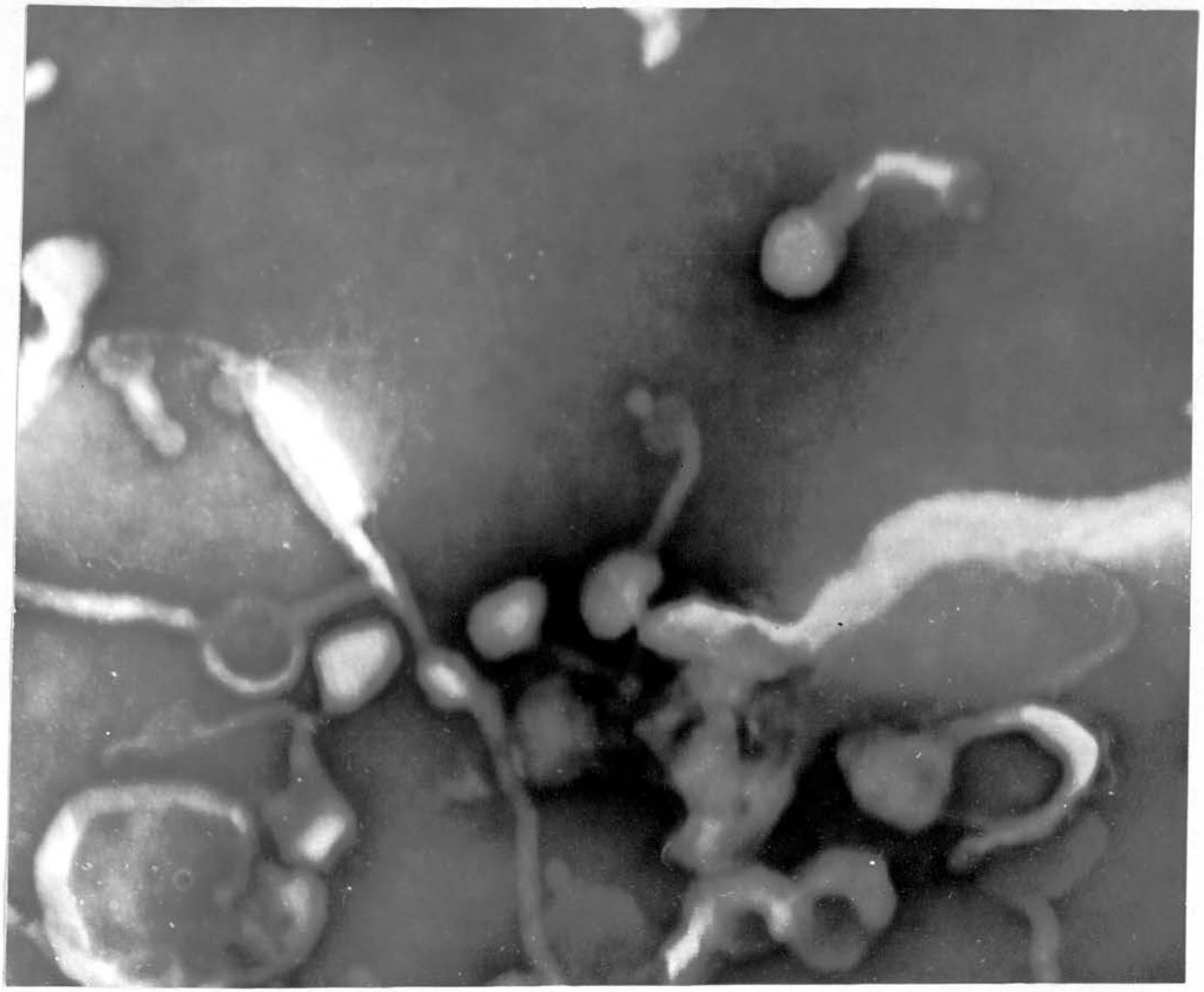


PLATE 14

Negatively stained preparation of partially purified osteopetrosis virus.

PLATE 14

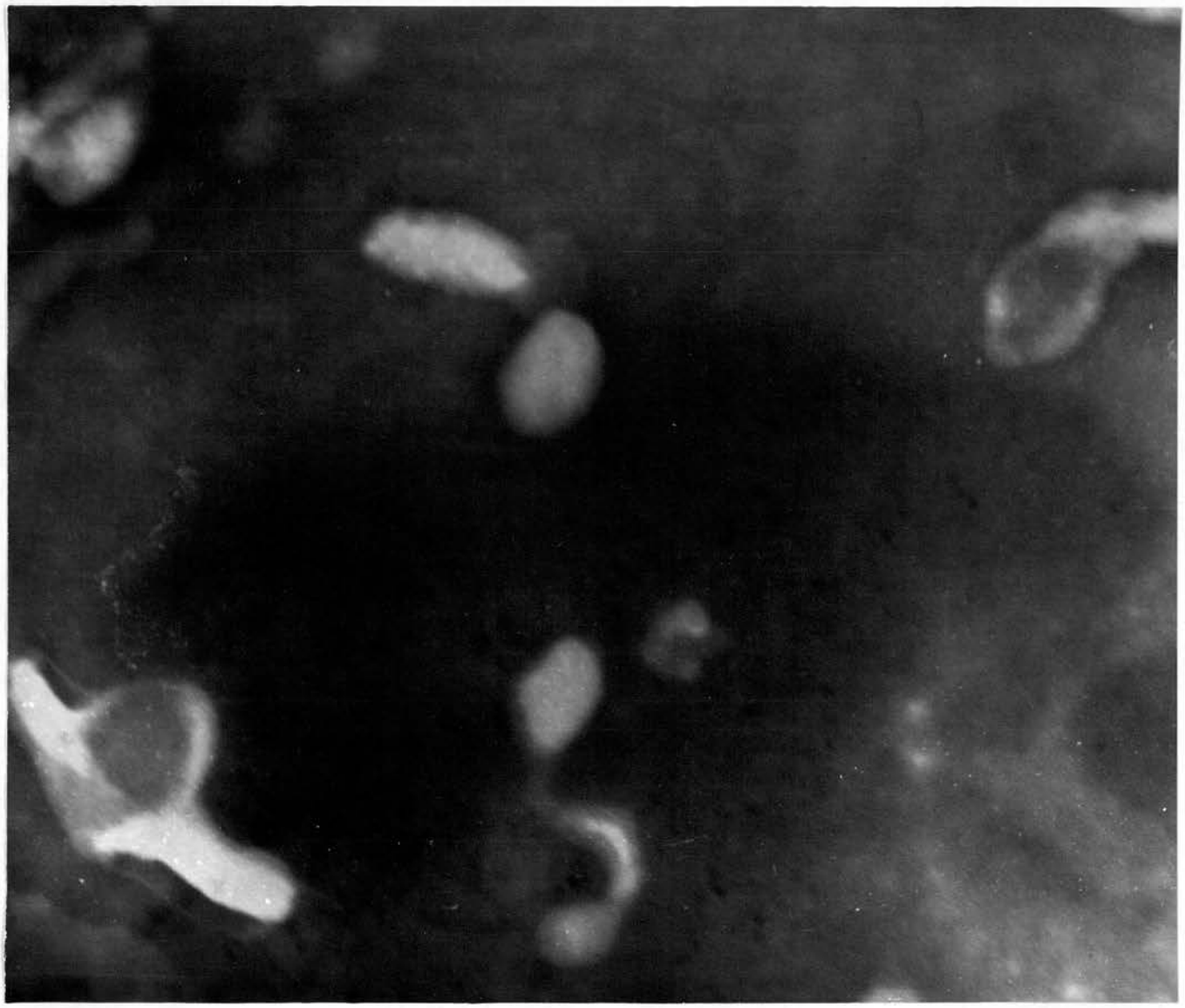


PLATE 15

R.N.A.-ase treated osteopetrosis virus particles in a section of infected kidney. The virus particles are situated in an intercellular space between two proximal tubule cells. The ribosomes and the nucleoids of the viruses no longer stain as densely as untreated R.N A.-containing structures.

PLATE 15

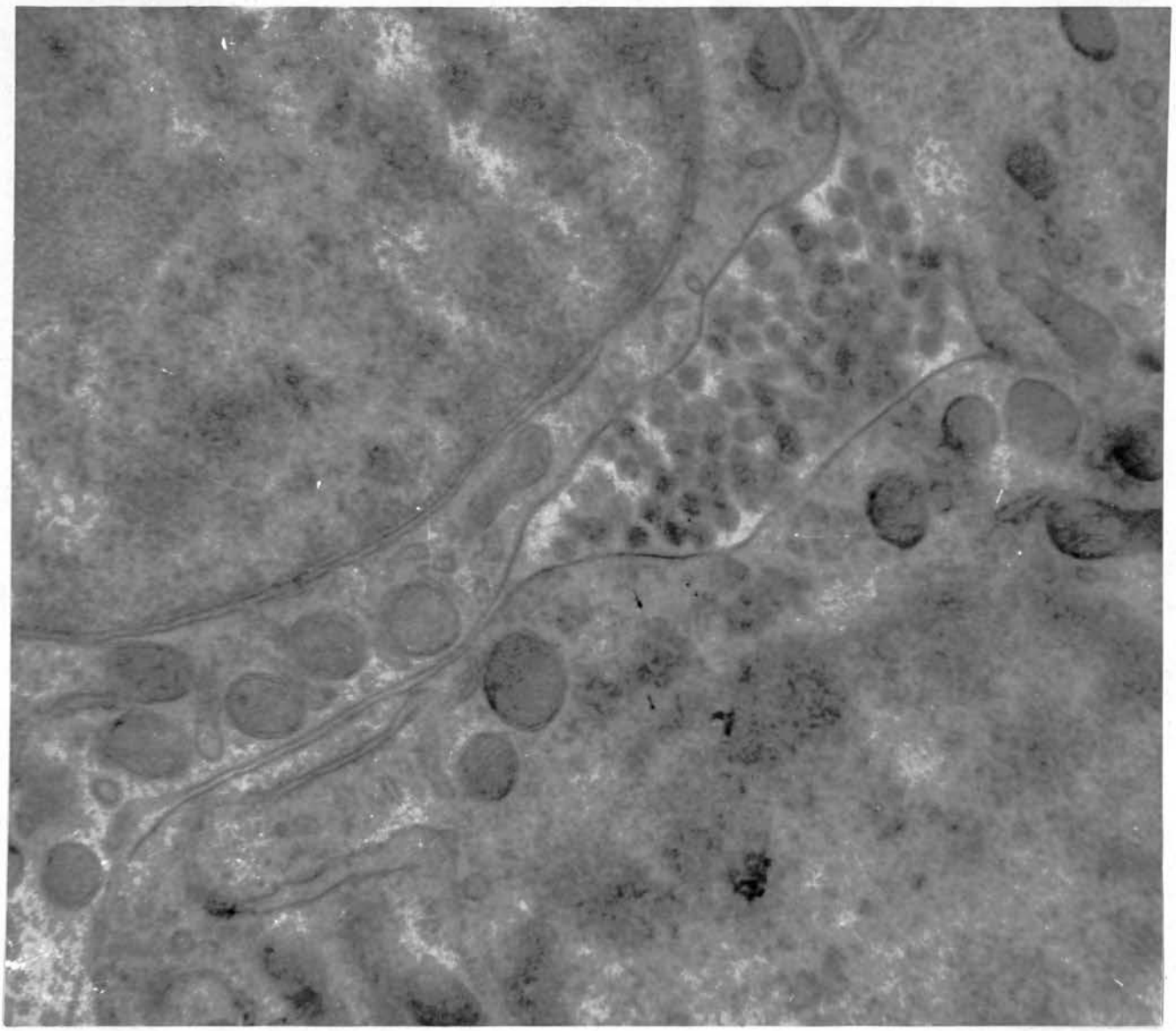


DIAGRAM I

Osteopetrosis virus.

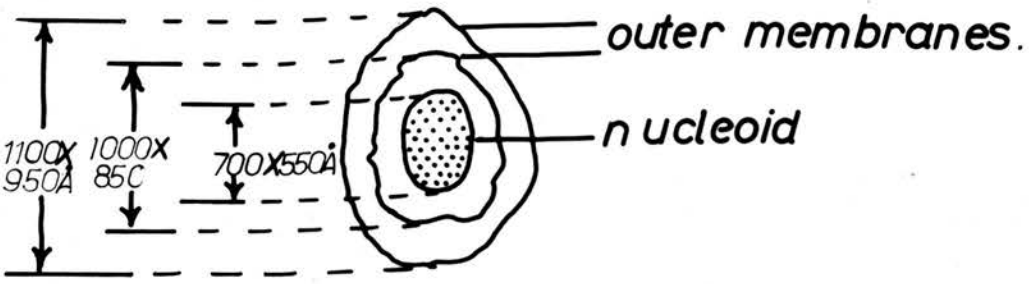


DIAGRAM II

This shows the variation in appearance of sections of the virus cut in different planes.

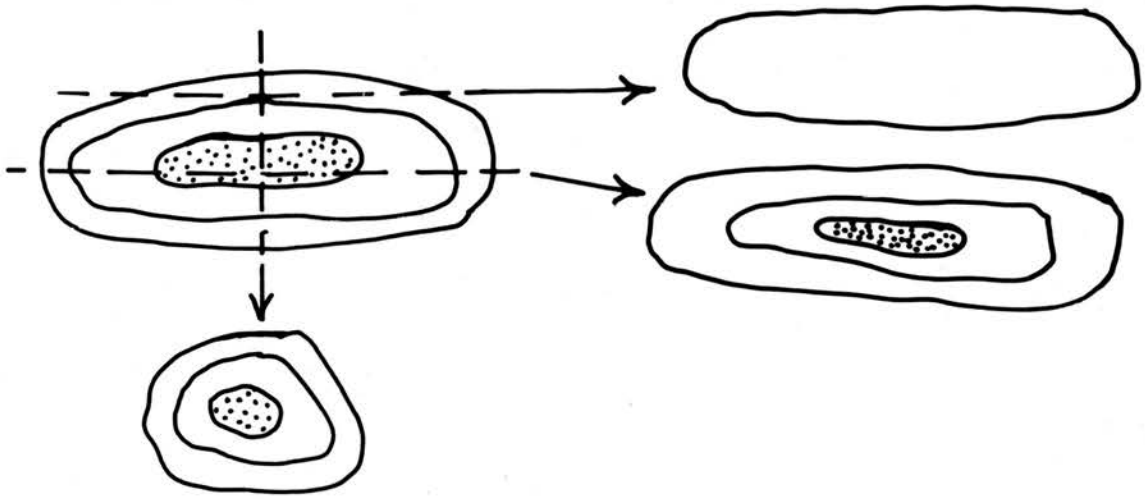


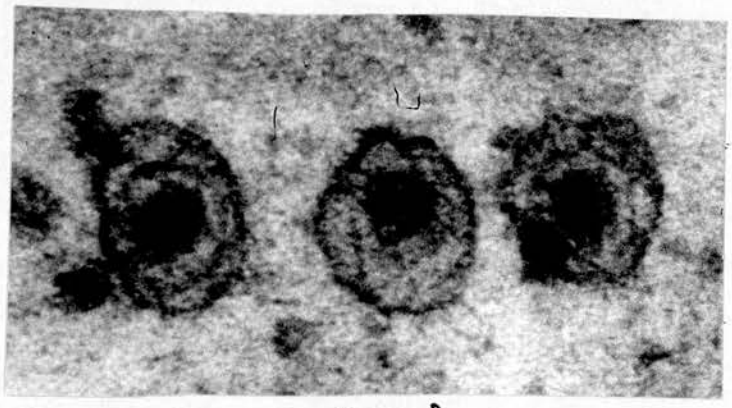
PLATE 16

This micrograph was taken in an A.E.I. E.M. 6. The central core of R.N.A. and the two outer membranes of the osteopetrosis virus particle can be seen.

PLATE 17

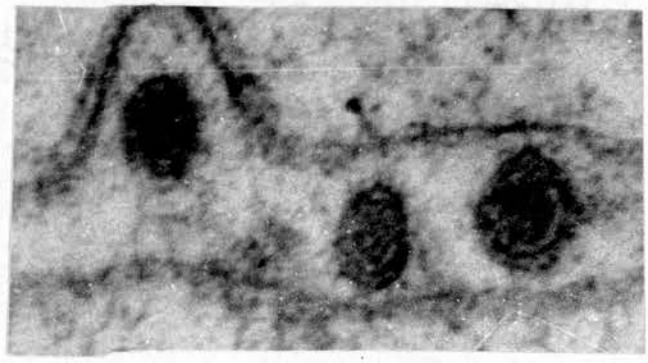
This micrograph was taken using a Phillips E.M. 100. The dense R.N.A. core and the lipoprotein membranes can be seen.

PLATE 16



1000Å

PLATE 17



1000Å

SECTION IV

ASSAY OF OSTEOPETROSIS VIRUS

INTRODUCTION

A considerable amount of time was spent in trying to grow viruses on the chorio-allantoic membrane (C.A.M.) and in tissue culture, but since the results were extremely inconsistent and in many cases not reproducible, this work is only mentioned briefly. In order to study the general growth pattern of osteopetrosis virus, it was thought necessary to find a way of cultivating the virus *in vitro*. A system in which the environmental factors can be closely controlled offers obvious advantages. In the case of the osteopetrosis virus, since growth in chickens appears to be so slow, both the cultivating and assaying of the virus *in vitro* would be very useful.

From the previous two sections of this work, it has been assumed that the osteopetrosis virus is probably like some of the other avian tumour viruses, in its characteristics. Consequently, some of the established methods of culture used for this type of virus were tried. R.S.V. itself was first grown on the C.A.M. of the chick embryo by Rous and Murphy (1912) and later by Keogh (1938). These observations were used by Rubin (1955), Prince (1958), and Vigier (1959) to develop quantitative assay techniques, depending on pock counts. Fairly accurate titre values can be obtained in seven days by titrating a virus extract onto the C.A.M.s of a group of eight embryos (Prince, 1958), whereas a titration by the limiting dilution method takes, in chickens, about thirty chickens and six weeks (Carr and

Harris, 1951). Tissue cultures of 9-day-old chick fibroblasts have also been used to grow Rous virus (Temin and Rubin, 1958). A similar type of virus assay to that of the C.A.M. assay can then be made with the cultured cells, since cells which become infected with Rous virus divide and produce characteristic 'mounds' of cells. If the infected cells are overlaid with nutrient agar, this assay is then an 'infective centre' count. R.S.V. can be grown quite easily in both tissue culture and on the C.A.M. MH_2 virus (Murray and Begg, 1930) and P.R.C. 4 virus (Carr and Campbell, 1958; Dhaliwal, 1959) have also been reported to produce pocks on the C.A.M. which can be used for assaying virus titres.

The leucosis viruses, specifically erythroleucosis, myeloid leucosis, and lymphomatosis, have all been reported as growing in tissue culture (Heine et al, 1961; Baluda and Goetz, 1961; Rubin, 1960), although the classification of the virus grown has occasionally been in doubt. These viruses have not been assayed by direct pock counts on the C.A.M., like the previously described ones, even though erythroleucosis has been reported to produce lesions on the C.A.M., (Campbell, 1963). A technique has been developed for assaying leucosis viruses, which depends on the interference of R.S.V. growth by the leucosis viruses, (Rubin, 1960). An assay of this type depends on the knowledge that three days or more after infection with leucosis viruses, cultures of chick fibroblasts become resistant to post-infection with R.S.V. This interfering characteristic has resulted in the phenomenon exhibited by the

leucosis viruses being called Rous-Interfering-Factor (R.I.F.). Vogt and Rubin (1963) showed that if a graph of serial dilutions of avian myeloid leucosis virus (A.M.V.) was plotted against the relative sensitivity of a constant titre of R.S.V., a linear relationship could be demonstrated. Another absolute method of initially titrating the virus was used giving a standard curve relating interference with the number of infectious units. In this case, the 'infectious unit' was defined as 'the smallest amount of A.M.V. sample still able to cause resistance to R.S.V. after being passaged through three sub-cultured cell generations'. Thus, by initially carrying out an end point titration of serial dilutions on a single preparation of A.M.V., a standard interference curve was produced. Subsequently, other unknown samples were used to obtain dose response curves which were compared with the standard curve. By a comparison of the two curves, the relative dilution factor could be measured from the graphs, and thus the actual titre in terms of infectious units determined. Osteopetrosis is morphologically similar to the leucosis viruses, and it was thought probable that it would behave in a similar way towards R.S.V. It was hoped that if osteopetrosis virus could be grown on the C.A.M. or in tissue culture, without pock formation, an interference technique of this type could be developed. This, then, was the object of the next part of the work.

CHORIO-ALLANTOIC MEMBRANE ASSAY

MATERIALS AND METHODS

Embryos used for inoculation of virus preparations were all 11-day-old embryos, obtained from the inbred flock of brown leghorns, selected for susceptibility to R.S.V. I, by Dr. John Carr, at the Poultry Research Centre. Virus preparations consisted of either:

1. crude suspensions of macerated cells, in 0.9% saline, which had been centrifuged at 1,000 r.p.m. to remove the larger cell debris:

2. partially purified preparations, as previously described, which were obtained from several different tissues.

Kidney and blood from birds suffering from osteopetrosis were used as virus sources. In some experiments, the kidneys, blood, and tumour extracts from birds which had been injected with P.R.C. 18 and which had developed osteopetrosis were used as sources. A description of P.R.C. 18 and its association with osteopetrosis can be found in section VI of this thesis. In each experiment, approximately six eggs were used for the assay of each virus preparation.

The virus preparations were titrated on the C.A.M.s of chick embryos, by a method similar to that suggested by Keogh, (1938). The eggs were arranged, embryo uppermost, swabbed with 70% alcohol, and then a triangular window cut in the shell with a dentist's drill. A hole was then drilled in the shell opposite the air space, and the C.A.M. 'dropped' by breaking

the egg membrane with a glass rod which had a rounded end.

0.1 ml. aliquots of the various osteopetrosis virus preparations were then dropped onto the C.A.M., and after the eggs had been sealed with selotape and wax, the embryos were left to incubate for seven days. In later experiments, after three days the eggs were opened and a quantity of R.S.V. added; giving a pock count of 50-100 / C.A.M. The eggs were then sealed again, and incubated for a further seven days, before pock counts were made. In all these experiments, when R.S.V. was injected into osteopetrosis infected eggs, equivalent aliquots from the same Rous preparation were injected into uninfected, normal eggs, as controls. The electron microscopy procedures used to examine the C.A.M.s were the same as those described in section II.

RESULTS AND DISCUSSION

Attempts to grow the virus. Virus preparations from the tissues known to be infected with osteopetrosis virus were injected onto the C.A.M.s. The results are summarised in table 5, page 93.

The response of the C.A.M. was scored as being positive or negative, and no quantitative values can be placed on these results. The positive response to the introduction of virus onto the C.A.M.s appeared to be a slight thickening, or an increase in the opacity, of the membrane. The response was uniform throughout the whole of the membrane, unlike the response to R.S.V., or erythroleucosis virus. Although the C.A.M. did show this effect, suggesting that the virus was growing and multiplying, the thickening and opacity was not

consistent in membranes of similar eggs treated with identical aliquots from the same virus preparation. There were also considerable variations in apparent effects on the C.A.M. of different preparations of the same tissue from different birds. This very poor 'take' on the C.A.M. was thought to have been due to the method of scoring. A C.A.M. was only considered to be infected if it showed a change in thickness or opacity. The virus could have been replicating without showing any obvious effect.

An examination of the injected C.A.M.s was undertaken, using the electron microscope, to determine whether or not the osteopetrosis virus was replicating, and to what extent.

Electron microscope examination of injected C.A.M.s. Initially, uninfected normal C.A.M.s were examined, to establish the complete absence of virus particles in all the controls. No virus-like particles were found.

The electron microscope revealed considerable numbers of virus particles in some of the osteopetrosis infected C.A.M.s examined three or four days after inoculation, and typically infected membranes are described in plates 18, 19, on pages 96,97 . All those membranes examined which were found to be infected with viruses had a high concentration of virus particles: no membranes were examined which had only very low concentrations. Romanoff (1960) describes the chorio-allantoic membrane of the chick embryo as consisting of two limiting layers of ectodermal cells and endodermal cells, separated by a layer of mesodermal cells. These three layers are easily distinguishable, and a transverse section of the

membrane can be orientated, since the endodermal cells have microvilli on their surfaces, which protrude into the allantoic cavity, (Borysko and Bang, 1953). The virus infection does not seem to be confined to any one layer of cells, and all three cell layers appear to have the ability to replicate virus particles. Virus multiplication appears to be like that found in infected tissues of the fowl (section II), and all the particles observed in the C.A.M. were extra-cellular.

From these observations, it is obvious that virus infection of the C.A.M. can be recognised definitely by this technique. Consequently, an examination of injected C.A.M.s was undertaken to determine the number of C.A.M.s infected, after injection, when scored on the basis of presence or absence of particles. These experiments were rather lengthy, as the fixation, embedding and sectioning techniques for the electron microscope took several days to complete, for each sample examined. Table 5, on page 93 summarises the results obtained.

It is obvious that the infection of the C.A.M. was of a very low order, and, in fact, the results obtained by this method were very similar to those obtained by the original crude scoring technique. This inconsistency of infection was rather disappointing, and is unexplained, although it has been reported that the growth of viruses in culture, particularly influenza virus, can be dependent on very specific environmental conditions. The exact conditions for optimum growth of osteopetrosis in the C.A.M. may not have been determined. Different egg lines were tried, but gave no improvement of

'take', and various different methods of virus preparations, including concentration by ultra-centrifugation and variation in suspension media were tried, with no improvement. However, even though these results did not indicate a uniformity of virus infection, it was thought that a comparison of the proportion of C.A.M.s infected, determined by the electron microscope examination, and the proportion infected, measured by an interference technique, might show comparable results. The possibility of osteopetrosis virus interfering with R.S.V. could be determined by this technique.

Rous sarcoma virus (R.S.V.) interference assay. The C.A.M.s were infected according to the previously described techniques with, first, osteopetrosis virus, and then R.S.V. The results of these experiments are summarised in tables 6 and 7 on pages 94 and 95. It can be seen from these results that there is a considerable variation of the pock counts, within identical samples, in C.A.M.s infected with both osteopetrosis virus and R.S.V. In some cases, there is complete and absolute interference with R.S.V., (experiment V), but it appears that there may be partial interference, too, (experiments VI, VII). This variation not only is in evidence between samples from different birds, but also between samples from the same tissue preparation.

The response of the eggs to R.S.V. titrations was constant, since the pock counts gave little variations between eggs injected with identical aliquots of the same R.S.V. preparations. Different lines of eggs were tried, but no

preferential susceptibility to osteopetrosis virus was shown by any particular line. Different methods of preparing the virus, as described on page 87, were also tried, but the variation was not reduced.

These results have shown that osteopetrosis virus can be grown on the C.A.M., and that high concentrations of virus can occasionally be obtained. On numerous occasions, absolute interference of R.S.V. growth was observed, resulting in the reduction of the R.S.V. assay to zero. However, the inconsistency of osteopetrosis virus growth on the C.A.M. does not permit an interference assay, which is dependent on an apparent reduction of an R.S.V. titre, to be carried out.

TISSUE CULTURE

INTRODUCTION

An attempt was made to grow osteopetrosis virus in tissue culture. The work of Rubin (1960) has already been described, and the techniques suggested by him formed the basis for the method tried. There have been many reports of the growth of leucosis viruses, either R.I.F. (Rubin, 1960), A.M.V. (Vogt and Rubin, 1963), or erythroleucosis (Lagerlöf, 1960), in cultures of fibroblasts. However, any assay based on changes by leucosis virus in the injected cells has always been by an interference method. On the other hand, confluent monolayers of cultured kidney cells have been reported to be transformed by A.M.V. (Baluda, 1962), and it was hoped that, if confluent monolayers of chick embryo kidney cells could be obtained, then an infective centre assay could be developed.

MATERIALS AND METHODS

Throughout all the handling of cells, strict aseptic conditions were maintained. The area on which the manipulations took place was washed with 70% alcohol before use, and an ultra-violet light was left switched on immediately above the bench for several hours before the cells were prepared. All solutions were sterilised by filtration or autoclaving; glassware and instruments were sterilised by dry heat.

Preparation of fibroblast cultures. Twelve-day-old embryos were used as a source of material. The embryos were extracted from the eggs and placed in a 100 ml. syringe, with a wide needle attached. This syringe had a wire mesh at the bottom of the barrel, through which the embryos were pressed. This proved to be a most efficient way of macerating embryos, since all the larger pieces, the bones and cartilage, were left behind in the syringe. The suspension of cells was then washed twice with phosphate buffered saline (P.B.S.) and then suspended in 50 mls. of a 3% trypsin solution in a flask. This mixture was gently stirred with a magnetic stirrer for thirty minutes, after which the flask was removed from the stirrer, and the cells centrifuged at 500 r.p.m. for two minutes. After the mixture had been centrifuged, only single cells remained in suspension. These were decanted, washed twice in P.B.S. by centrifuging at 3,000 r.p.m., and resuspended in a mixture of 90% Difco 199 tissue culture medium and 10% chicken serum. (The serum was prepared by collecting the fluid from clotted blood, left for three hours at room

temperature: this fluid was then centrifuged at 5,000 r.p.m., and stored at 4°C.) The cell suspension was then counted in a haemocytometer, and diluted with the culture medium, Difco 199, to give a suspension of 4 x 10^6 cells/ml. This suspension was then distributed in 4 ml. of aliquots, to 5 cm. petri dishes, which were then placed in an air-tight perspex container. (See page XIX of the Appendix.)

A 5% CO_2 / 95% air mixture was then introduced into this container, which was then sealed up, and left at 37°C. in an incubator. After twenty-four hours, 0.5 ml. volumes of concentrated virus extracted from the blood or kidneys of birds infected with P.R.C. 18 tumours or osteopetrosis were then introduced into the cultures. The culture medium was removed before the virus was introduced, in order to ensure optimal conditions for absorption of virus by the cells.

Kidney cell preparations. Kidneys were disected out from 12 - 16-day-old embryos, and treated in the same way as described for fibroblasts.

RESULTS AND DISCUSSION

Fibroblast cultures. It was found that, if suitably high concentrations (4 x 10^6 / ml. x 5 ml.) of cells were used for initial seeding of the petri dishes, confluent monolayers were free from cell aggregates from undissociated pieces of tissue. The monolayers consisted virtually entirely of spindle shaped fibroblasts, and only very rarely were other cell types observed. These monolayers, prepared by this simplified

technique, were considered adequate for virus assaying. Cells were treated with virus extracts to determine if there were any morphological changes in the normal fibroblast structure or behaviour. After three to seven days, the virus infected cultures showed no change in morphology before, or after, staining as a monolayer. Unfortunately, normal cultures could not be maintained for periods of more than one week, without various inconsistencies between cultures arising. As a result of the difficulties encountered in maintaining cells in culture for more than seven days, this work was dropped.

Kidney cells. In attempts to grow kidney cells in cultures, confluent monolayers could not be obtained, using the same techniques as for chick fibroblasts. Kidney cells grow much more slowly than fibroblasts in culture, and the cells are much smaller in size. However, since the regions in which the kidney cells grew proved to consist of a uniform population of cells, several variations in technique were tried, to obtain confluent monolayers. Embryos of different ages were used as sources of material; P.B.S., 0.9% saline, and Difco 199, were each used separately for washing procedures. Various 'gentle' trypsinisation procedures were tried, using different calcium- and magnesium-free solutions with 0.1%, 0.5%, 1.0% trypsin. Disassociated cells were grown in Difco 199 + 5% serum, a 50/50 mixture of Difco 199 and serum, and serum with P.B.S. None of these modifications helped the cells to grow into monolayers. These cultures, like those of fibroblasts, could not be maintained consistently for more than seven days. This

work was also reluctantly dropped.

The difficulties encountered in culturing cells were of two types:

1. environmental difficulties, which were probably due to the lack of a suitable CO_2 incubator. The gas-tight containers designed to keep cells in a CO_2/air atmosphere were adequate for short term cultures, but required regular gassing with CO_2/air. The constant change in p.H. and temperature probably was not conducive to optimum growth of the cells;

2. contamination: a great deal of trouble was experienced with fungal contaminants, and although various fungacidal agents recommended by Dr. John Paul of the Biochemistry Department in Glasgow were tried, no adequate solution was found to this problem.

TABLE 5

INOCULATION OF OSTEOPETROSIS INFECTED BLOOD ONTO THE CHORIO-ALLANTOIC MEMBRANES (C.A.M.s) OF 11 DAY OLD EMBRYOS

Experiment	Source of Virus	Number of Embryos inoculated	Number of C.A.M.s infected scored by eye	% infected
I	Whole blood from florid cases of osteopetrosis	106	16	15.1
	Whole blood from P.R.C. 18 infected birds	109	24	22.0

Experiment	Source of Virus	Number of Embryos inoculated	Number of C.A.M.s infected scored by E.M. examination	% infected
II	Whole blood from florid cases of osteopetrosis	53	5	9.4
	Whole blood from P.R.C. 18 infected birds	46	7	15.4

TABLE 6

INOCULATION OF OSTEOPETROSIS VIRUS PREPARATIONS ONTO THE C.A.M.

FOLLOWED BY ROUS SARCOMA VIRUS (R.S.V.) PREPARATIONS

Experiment	Source of osteo-petrosis virus	Number of eggs inoculated with osteopetrosis virus and R.S.V.	Mean number of Pocks / C.A.M.	Number of eggs inoculated with R.S.V. alone	Mean number of Pocks / C.A.M.
III	P.R.C. 18 blood	15	44.3	10	46.4
	Kidney from osteo infected birds	15	40.2		
IV	P.R.C. 18 blood	12	75.1	9	83.2
	Kidney from osteo infected birds	12	77.9		

TABLE 7

INOCULATION OF OSTEOPETROSIS VIRUS PREPARATIONS ONTO THE C.A.M.s OF 11 DAY OLD EMBRYOS FOLLOWED BY R.S.V.

Experiment	Number of eggs inoculated with osteopetrosis followed by R.S.V.	Number of pocks	Mean	Number of eggs inoculated with R.S.V. alone	Number of pocks	Mean
V	9	0 0 0 0 0 0 0 0 0	0	10	56 53 57 55 54 54 55 55 56 58	54.8
VI	12	84 80 69 82 86 52 0 83 80 81 81 0	65.08	6	86 90 83 81 84 85	84.1
VII	6	103 112 90 97 99 120	103.5	6	109 115 119 120 93 99	109.1

Interference expressed as: $100 \times \frac{\text{difference of experimental and control pock counts}}{\text{number of pocks in control}}$ %

Experiment V	Experiment VI	Experiment VII
100%	20.83%	5.18%

PLATE 18

Transverse section of a C.A.M. infected with osteopetrosis viruses which can be seen in the intercellular space.

PLATE 18

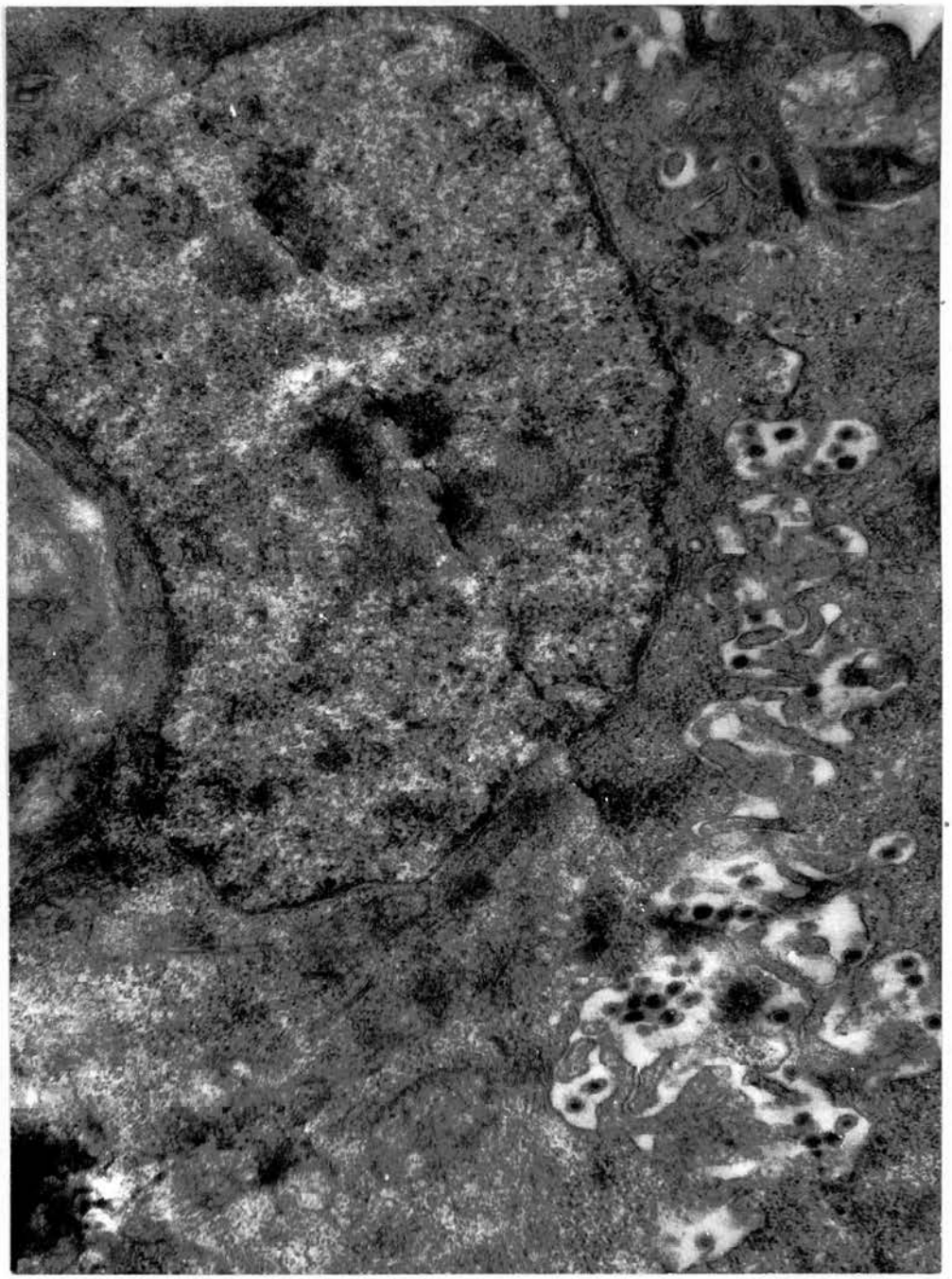
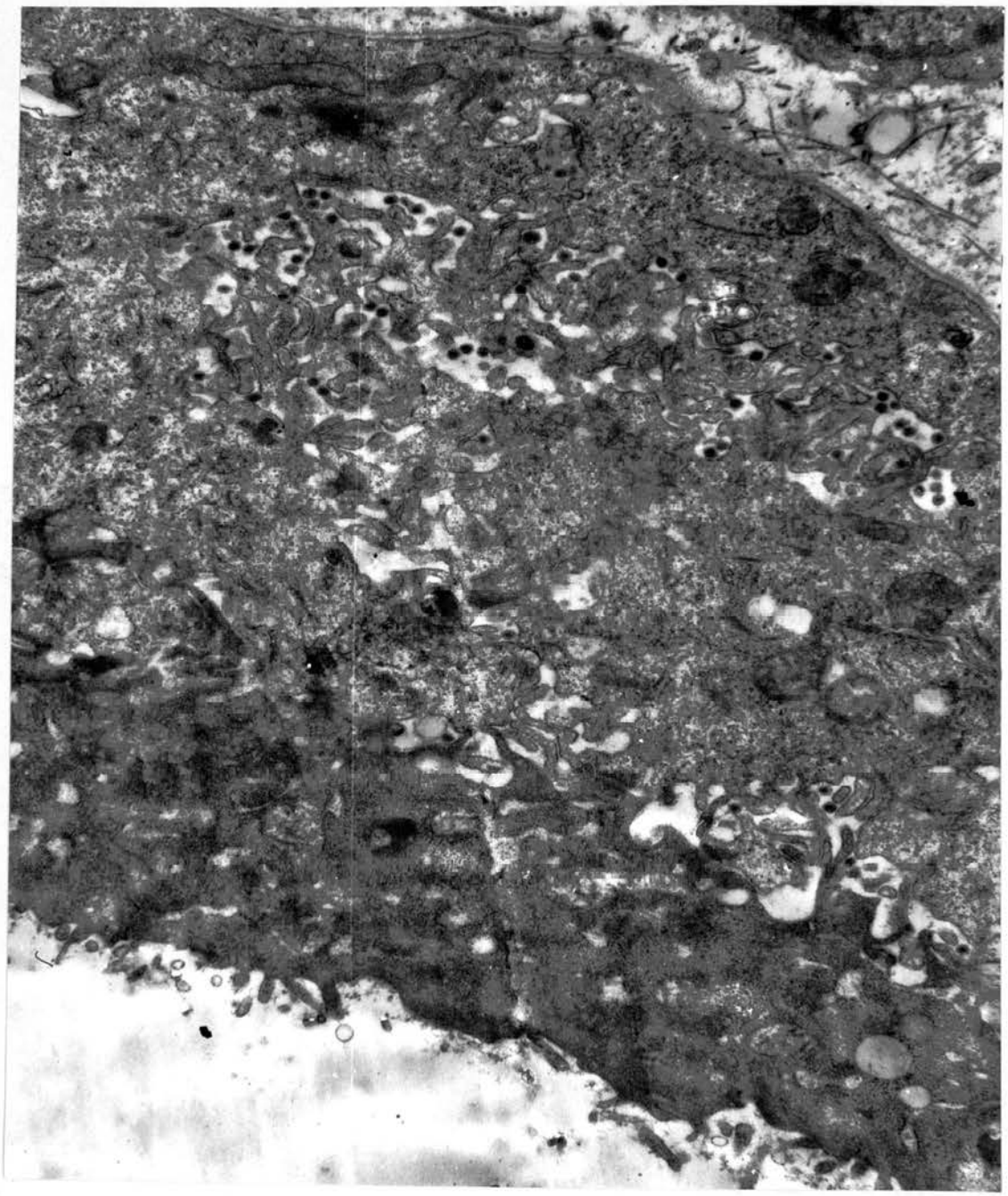


PLATE 19

Transverse section of a C.A.M. infected with osteopetrosis viruses. The endodermal cells can be seen at the lower surface with microvillii protruding into the allantoic cavity.

PLATE 19



SECTION V

LIFE CYCLE OF THE OSTEOPETROSIS VIRUS IN CHICKENS

PART I: ORGAN INVOLVEMENT

INTRODUCTION

Having established that a particle similar to the Rous Sarcoma Virus (R.S.V.) was responsible for osteopetrosis, an attempt was made to follow the development of the disease and to determine the sequence of organ involvement, particularly at an ultrastructural level. Since the attempts at finding an *in vitro* assay of the osteopetrosis virus failed, the only available alternative method of detecting the virus was by examining tissue thought to be infected, with the electron microscope. This detection is not quantitative, but is probably capable of distinguishing between tissues which are infected and those which are not. In this investigation, since the assay of virus by chicken inoculation was far too slow, and culture methods had not been developed, the electron microscope served as a very useful tool in that it was the only way of demonstrating the presence or absence of osteopetrosis virus.

It is extremely difficult to find references to the complete life histories of the leucosis viruses in chickens, although many workers have reported the involvement of pancreas, lymphoid tissue, spleen and marrow, and suggested the kidney, (Burmester, 1959b; Dmochowski et al, 1961). Beard (1963) suggests that infection of the pancreas, in viraemic, but not leucaemic, birds can result in reservoirs of virus which can explain the only too well known epidemics of fowl leucosis,

which occur even in the best maintained, and even isolated, flocks. It was hoped that an examination of birds at different ages, after infection of osteopetrosis virus at one day old, would show the centres of virus replication.

It has often been reported that osteopetrosis has a predeliction for the male birds, rather than the females. Occasionally, females do develop osteopetrotic lesions, as mentioned in section II, but less frequently than males. It was considered that an examination of all birds which had originally been infected with osteopetrosis preparations, irrespective of whether or not they developed the associated lesions, might indicate the possibility of some birds being carriers, or at least having the type of 'virus reservoirs' suggested by Beard.

MATERIALS AND METHODS

One-hundred, day-old chicks were injected intramuscularly with 0.2 mls. of blood from birds showing clinical signs of osteopetrosis. Fifty birds were injected at the same age with 0.2 mls. of 0.9% saline. Between six and fourteen birds, in the approximate ratio of two infected to one control, were killed each week for a period of twelve weeks. Specimens of kidney, spleen, pancreas, liver, periosteum and marrow were examined with the electron microscope, after fixation, embedding and sectioning, (as described in section II). The specimens from injected and uninjected birds were then examined and compared.

RESULTS

All the tissues were examined with the electron microscope to determine whether or not they contained mature virus particles. Although the presence of one particle per section would have been considered sufficient to conclude that the particular tissue was infected, sections with single particles were never found. Tissues that were scored as 'infected' contained numbers of viruses comparable to those of the tissues from adult florid cases (examined in section II of this work). The observations are summarised in table 8 on page112 . It is interesting to compare the times at which virus is first observed in the tissues. Virus was first found in the kidney after about three weeks. This was the first organ to show a virus infection after injection at one day old. Next, the spleen and marrow were found to have virus at about four weeks old, and then very occasional virus particles were found in the liver after six weeks. Virus particles were also observed in the pancreas after six weeks, and, finally, virus particles were demonstrated in the periosteum at eight weeks.

Testes and ovary involvement was found only in older birds which were not less than ten weeks old. The muscle lesions described in section I were found only in birds which were not less than six months old, and, even then, the incidence was very low (see table 2, page 33).

Although the virus could not be assayed, and a relative quantitative estimation of virus in the different infected tissues obtained, the electron microscope showed, at all times,

much greater concentrations of virus in the kidney than in any other tissue examined. About three weeks after infection, the kidney appears to be the only site at which virus can be found. 90.5% of male and female birds, examined more than four weeks after injection of osteopetrosis virus, were found to have virus in the kidney. There was only a very slight sex difference in the infection of the periosteum, since more males appeared to have viruses in this tissue (see table 9, page 113). However, the time at which the male and female periosteum becomes infected appears to be approximately the same, although this is not significant, since such a small sample of birds was examined.

One particularly interesting, though isolated case, appeared in a young chick, only one week old. The bird was killed, although it looked in good health, and was found to have a small kidney tumour, which contained osteopetrosis-like particles.

DISCUSSION

These experiments show progressive infection of the various organs of the birds which were examined. Virus particles were never found in any of the tissues of the control birds. The presence of virus particles was first observed in the kidneys of the infected birds, which suggests that this organ may be the primary centre of virus replication. There are several other pieces of evidence which support this contention.

In one case examined, a one-week-old bird was found to have a virus associated tumour in the kidney. This tumour occurred

well before virus was found in any of the other organs, and suggests an early accumulation of virus at this site.

Another situation which suggests that the kidney is the primary centre of osteopetrosis virus replication, is the kidney infection within a week of the injection of P.R.C. 18 into day-old chickens. (The characteristics of P.R.C. 18 are reported rather more extensively in section VI.) The P.R.C. 18 tumour is heavily contaminated with osteopetrosis virus, consequently, as the tumour develops, the organ, or tissue, with the greatest susceptibility to the virus might be expected to be the first to show demonstrable virus and virus replication.

One of the original assumptions in this work has been that, since no other virus associated leucotic condition has been manifested in the routine passaging of the osteopetrosis virus, and since the flock maintained at the Poultry Research Centre has always been free of leucosis, the osteopetrosis virus strain is genetically homogeneous. Consequently, as no viruses have been found in any of the control birds, then all viruses in the experimental birds are likely to be the osteopetrosis viruses. As the virus concentration in the kidney is higher than that found in any other organ, it is likely that this organ is a source of dissemination to the other organs.

Many other viruses are known to have an affinity for kidney cells, (polio virus, Mayor and Jordan, 1962; myeloid leucosis (B.A.I. strain A.), Thorell, 1958a; erythroleucosis, (E.S.4 strain), Carr, 1959; and infectious bronchitis, Siller 1964), it is not, therefore, surprising that the virus

responsible for osteopetrosis should show an affinity for this organ.

The subsequent infections of the spleen, marrow, pancreas, ovary and testes, periosteum and muscle cells, suggest that the virus has entered the blood and is being disseminated throughout the body. This does not explain the infrequent infection of the periosteum, resulting in the diagnostic feature of the disease. Hormonal factors may be responsible for the higher incidence of bone lesions encountered in male birds, but this possibility was not investigated. As tissue culture facilities and assay techniques were not available, an investigation of the parameters would have been difficult.

Since it has been assumed that the viruses found throughout all tissues of the infected birds were identical, it can be seen from table 8, page 112, that 90.5% of the birds injected with osteopetrosis became viraemic, but only 16.9% osteopetrotic. In this respect, male and female birds do not appear to vary in their susceptibilities: only 9.5% of the inoculated birds were found to be completely uninfected.

As a result of these observations, three further lines of investigation were undertaken.

PART II: VIRUS GROWTH IN THE KIDNEY

INTRODUCTION

Virus was isolated from kidney tissue in cell-free suspensions, in order to determine the pattern of infection of virus obtained from this source.

Secondly, urine was obtained in order to determine the infectivity of the particles observed in the lumens of the kidney tubules. This could be a possible route for natural infection.

Finally, a detailed examination of the kidney was carried out, with the electron microscope, to investigate the exact distribution of the virus, and the changes occuring in the kidney, during the course of infection.

MATERIAL AND METHODS

Kidneys from birds with advanced osteopetrosis lesions were dissected out, and cell-free suspensions prepared by macerating the tissue in distilled water. The cellular debris was then removed by centrifugation at 1,500g. 0.2 ml. aliquots of this suspension were then injected into day-old chicks. Control experiments were carried out, using uninfected kidneys.

Urine was obtained from birds with advanced osteopetrotic lesions. These birds were first tied securely to a wooden frame and cotton wool was inserted through the vent into the cloaca, so that the urodaeum was clear of faeces. A small plastic funnel was then inserted into the cloaca and held in place by means of strips of elastoplast. Birds treated in this way were then left covered with a cloth, in order to keep them quiet, and urine, uncontaminated with faeces, was collected in test tubes. Day-old chicks were then given 0.5 ml. doses of urine orally, or 0.2 mls. aliquots intra-muscularly. Control experiments were carried out using uninfected birds as sources

of urine.

The detailed examination of the distribution and development of viruses in the kidney was carried out on material previously obtained from the experiment in which day-old birds were infected with osteopetrosis and killed at intervals of a few days.

RESULTS

Birds infected with kidney extracts. A survey of the distribution of virus particles, and the lesions obtained from the injection of these preparations, is summarised in table 10. The pattern of infection seems to be very like that found in birds injected with osteopetrotic blood: a low incidence of bone and muscle lesions, but a high incidence of kidney infection. The kidney extracts must certainly have contained blood borne virus, but a high percentage of 'kidney virus' must also have been present. Kidneys of birds infected with these preparations did not show unusually high concentrations of virus particles, compared with those of osteopetrotic birds from routine passages, and neither did they show a high incidence of kidney tumours.

Birds infected with urine. Table 11, page115 , summarises the effect of the inoculation of infected urine into day-old chicks. Oral administration results in a low incidence of virus infection, and only one in twenty birds developed osteopetrosis, although four developed kidney infections. However, six out of twelve day-old chicks which survived intra-muscular

inoculation developed kidney infections, of which three also had periosteal lesions.

The course of infection in the kidney. Virus particles were first found in the kidney after about three weeks. At this time they were very few in number, and only in proximal regions of the nephron, and in the glumerulus. There was a progressive build up of virus in the region of the epithelial cells of the tuft, and at about four weeks, frequent budding was seen from the cell-membranes of these cells, (plates 3, 21, 22, pages 36, 117, 118). This gradual increase in virus production by the epithelial cells appeared to reach a maximum at about four to five weeks, and after this time no further increase in concentration could be detected. During this period (age three to four weeks), the intra-tubular urine was becoming more and more heavily contaminated with virus particles. The cells lining the tubule were found to be intimately associated with viruses after the fourth week. Different regions of the tubules could be distinguished easily by the morphology of the cells that lined them, (Rhodin, 1963a), (plate 26, page 122). Consequently, it was relatively easy to follow the progressive infection of cells further and further down the tubule. After the fourth week, virus particles could be seen between adjacent cells of the proximal region of the tubules, and at the fifth week, distal tubule cells showed virus particles between their interfaces, (plates 24, 25, pages 120, 121). The collecting tubule cells did not appear to be infected to the same extent as the proximal and distal regions, and only very occasionally

were virus particles seen between cells of the collecting tubules. The infection of the distal and proximal region eventually led to large concentrations of virus at the bases of the tubule cells, between the cell membranes and the basement membrane, surrounding the whole kidney tubule (plate 30, page 126). At about five to six weeks, these concentrations were quite large, and at six weeks, virus particles were frequently seen in the blood, in regions immediately adjacent to these large concentrations of virus (plate 31, page 127). Virus particles were occasionally seen actually in the basement membranes, at about five to six weeks, opposite distal and proximal cells (plate 33, page 129).

Pinocytosis was observed at the cell surface of kidney cells, adjacent to the lumen, in the distal and convoluted regions. Although virus particles were never seen being absobed by cells, it is probable that this is a route by which viruses can enter and infect kidney tubule cells (plate 23, page 119).

Tubular hypertrophy, and cysts which were occasionally lined with cilia, were infrequently observed, but these conditions were found only in older birds, six months to a year after initial infection with osteopetrosis (plates 34, 35, pages 130, 130).

DISCUSSION

Day-old birds, infected with extracts from osteopetrosis infected kidney tissue, appeared to develop osteopetrotic

symptoms in a way similar to those infected with whole blood. This does not confirm that the virus replicating in the kidney was capable of producing osteopetrosis, since this tissue contains a lot of blood, and blood itself is a good source of infective virus. Consequently, the kidney extracts would contain blood borne viruses. However, no significant increase in the degree of kidney involvement was noticed in birds infected in this way. This suggests that the high titre of the virus produced from the kidney cells, found in the kidney extracts, merely produced the normal osteopetrotic condition. This supplied further proof that the virus growing in the kidney is the same as that responsible for osteopetrosis.

Both the oral and the intra-muscular injections of day-old chicks with urine from osteopetrotic birds resulted in virus infections. This also indicates that the virus particles in the urine were infective osteopetrotic particles, and not merely viruses with morphological characteristics similar to those of the osteopetrosis particles. As might be expected, infection by the oral route proved less successful than the intra-muscular route. However, the fact that the urine could infect chickens by these routes suggests that natural transmission could occur from the contamination of food stuffs, or drinking water. Infective urine, and infected reproductive organs, are probably responsible for the horizontal and vertical transmission of this disease in the field, respectively.

DISCUSSION

Since the object of this work has been to follow the course of infection of the osteopetrosis virus, cilio genisis has not been considered in detail, but Sorokin (1962) gives an excellent review of the subject.

The course of infection in the kidney. The kidney is probably infected by the haematogenous route. Virus particles enter the blood after inoculation or possibly after absorption by the cells of the alimentary tract. These particles are then carried to the glomerulus, where they are actively passed through the basement membrane, from the blood capillary to the urinary space. Rhodin (1962, 1963), in general reviews of the kidney structure, and Pak Poy and Robertson (1957), in an examination of the avian renal glomerulus, have shown that, at this point, the only continuous layer separating the blood and the urine is the basement membrane. The endothelial cells of the blood capillaries have many small fenestrations, so that these cells do not form a continuous layer over the lining of the capillary. Similarly, the epithelial cells have a cytoplasm which extends over the capillaries in long slender processes (plate 20, page 116). Several authors have shown that particles between 50Å and 2-400Å in diameter, e.g. ferritin, thorotrast, colloidal gold, and dextrans, are rapidly passed through the basement membrane of the glomerulus, after being injected into the blood, and are passed through the urinary space (Sampaio, 1956; Hall, 1957; Farquhar and Palade, 1959, 1960,

1961, 1962; James and Ashworth, 1961). Eventually, these particles, which are absolutely biologically inert, pass into the lumen of the kidney tubule, but are occasionally phagocytosed by the epithelial cells in the glomerular region, or the kidney cells lining the tubule (plate 23, page 119).

The passage of virus particles through the basement membrane may be active or passive: this question is open. However, virus particles have been seen at this point, actually in the middle of the basement membrane. As a result of Palade's work, the knowledge that viruses appear to concentrate at this point, the fact that the kidney actively 'filters' blood, and that viruses have never been seen in the blood in this region, it was concluded that the passage of virus is unidirectional and centrifugal in the glomerulus. The virus then infects the visceral epithelial cells of the glomerular tuft, probably by a process similar to the absorption of inert particles, as described by Farquhar and Palade (1962). Budding in great profusion takes place here. Consequently, a large amount of virus is produced in the Bowman's capsules, and is liberated into the urinary space, and then transported in the urine down the lumen of the tubules (plates 27, 28, 29, pages 123, 124, 125).

Infection of the distal and proximal tubules cells is probably by a process of pinocytosis. The infected cells then replicate virus particles between adjacent cells, and the surrounding basement membrane. Virus which is not shed into the inter-cellular spaces is probably unable to pass into the

lumen, since the desmosomes form an inpenetrable 'joint' preventing their passage. The function of desmosomes is to produce a continuous cellular layer to allow isolation of excretion products in the lumen and prevent their diffusion back into the blood. The virus particles in these regions then pass through the basement membranes surrounding the tubules, and move into the blood. This process is assumed to be 'one way' centripetally, and in the reverse direction to that found in the glomerular region, for two reasons.

1. Active diffusion of material back into the blood is not opposed by a movement of material in the reverse direction at this point.

2. Virus particles are observed in small groups in the blood opposite large concentrations of virus particles, and this is never seen in the glomerular regions.

As a result of these conclusions, a diagrammatical representation of kidney infection is described on page 131 of this work, and a summary of the proposed natural life cycle is on page 132 .

TABLE 8

DISTRIBUTION OF OSTEOPETROSIS VIRUS PARTICLES IN INFECTED BIRDS

	Age (in weeks) at death													
	1	2	3	4	5	6	7	8	9	10	11	12	Total	% of Total
Number of birds killed	6	6	6	7	7	7	6	7	6	8	8	14	89	100.0
Birds with virus in:														
Periosteum	0	0	0	0	0	0	0	2	1	3	4	5	15	16.9
Muscle lesions	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0
Marrow	0	0	0	1	1	0	2	0	0	1	1	2	8	9.0
Spleen	0	0	0	1	2	3	3	3	3	4	2	5	26	29.2
Pancreas	0	0	0	0	0	2	3	3	3	4	3	4	22	24.7
Liver	0	0	0	0	0	1	0	0	0	1	0	0	2	2.3
Ovary	0	0	0	0	0	0	0	0	0	0	1	2	3	3.4
Testes	0	0	0	0	0	0	0	0	0	1	1	1	3	3.4
Kidney	1	0	4	4	6	7	5	6	6	7	8	13	67	75.5
Kidney abnormalities:														
Cilia	0	0	0	0	0	0	0	0	1	0	1	2	4	4.5
Tumour	1	0	0	0	0	0	0	0	0	0	0	0	1	1.1
Cyst	0	0	0	0	1	2	2	2	1	2	3	2	15	16.9

%age of all inoculated birds with viruses in the kidney at 5 weeks or later = 90.5

%age of all birds inoculated, viraemic but not osteopetrotic = 74.0

TABLE 9

DISTRIBUTION OF OSTEOPETROSIS VIRUS PARTICLES IN MALE AND FEMALE BIRDS AT DIFFERENT AGES

	Age (in weeks) at death												Total	% of number killed
	1	2	3	4	5	6	7	8	9	10	11	12		
MALES														
Number of birds killed	4	3	4	4	3	5	3	3	4	4	5	8	51	100.0
Birds with viruses in periosteum	0	0	0	0	0	0	0	2	0	2	1	4	9	17.6
Birds with viruses in kidney	1	0	2	2	3	4	4	2	4	4	5	8	39	76.5
FEMALES														
Number of birds killed	2	3	2	3	4	2	3	4	2	4	3	6	38	100.0
Birds with viruses in periosteum	0	0	0	0	0	0	0	0	1	1	3	1	6	15.8
Birds with viruses in kidney	0	0	2	2	3	3	1	4	2	3	3	5	28	73.6

TABLE 10

DISTRIBUTION OF OSTEOPETROSIS VIRUSES IN BIRDS INOCULATED WITH CELL-FREE EXTRACTS OF KIDNEYS FROM BIRDS WITH OSTEOPETROSIS

	Extracts from infected kidneys	Extracts from normal kidneys
Number of birds inoculated	20	8
Birds with virus in:		
kidney	16	0
periosteum	4	0
Kidney abnormalities:		
cysts	6	0
tumours	0	0
cilia	3	0

All birds were killed at about twelve weeks.

TABLE 11

DISTRIBUTION OF OSTEOPETROSIS VIRUSES IN BIRDS

INOCULATED WITH URINE FROM BIRDS WITH OSTEOPETROSIS

	Oral inoculation	Intramuscular inoculation	Intramuscular inoculation of normal urine
Number of birds inoculated	20	19	9
Birds with virus in:			
kidney	4	6	0
periosteum	1	3	0
Number of birds dead of unknown cause after one day	2	8	4

All birds were killed between twelve and sixteen weeks.

PLATE 20

Part of the glomerular region of the kidney nephron. This micrograph shows that the only continuous layer between the blood and the urine is the basement membrane.

PLATE 20

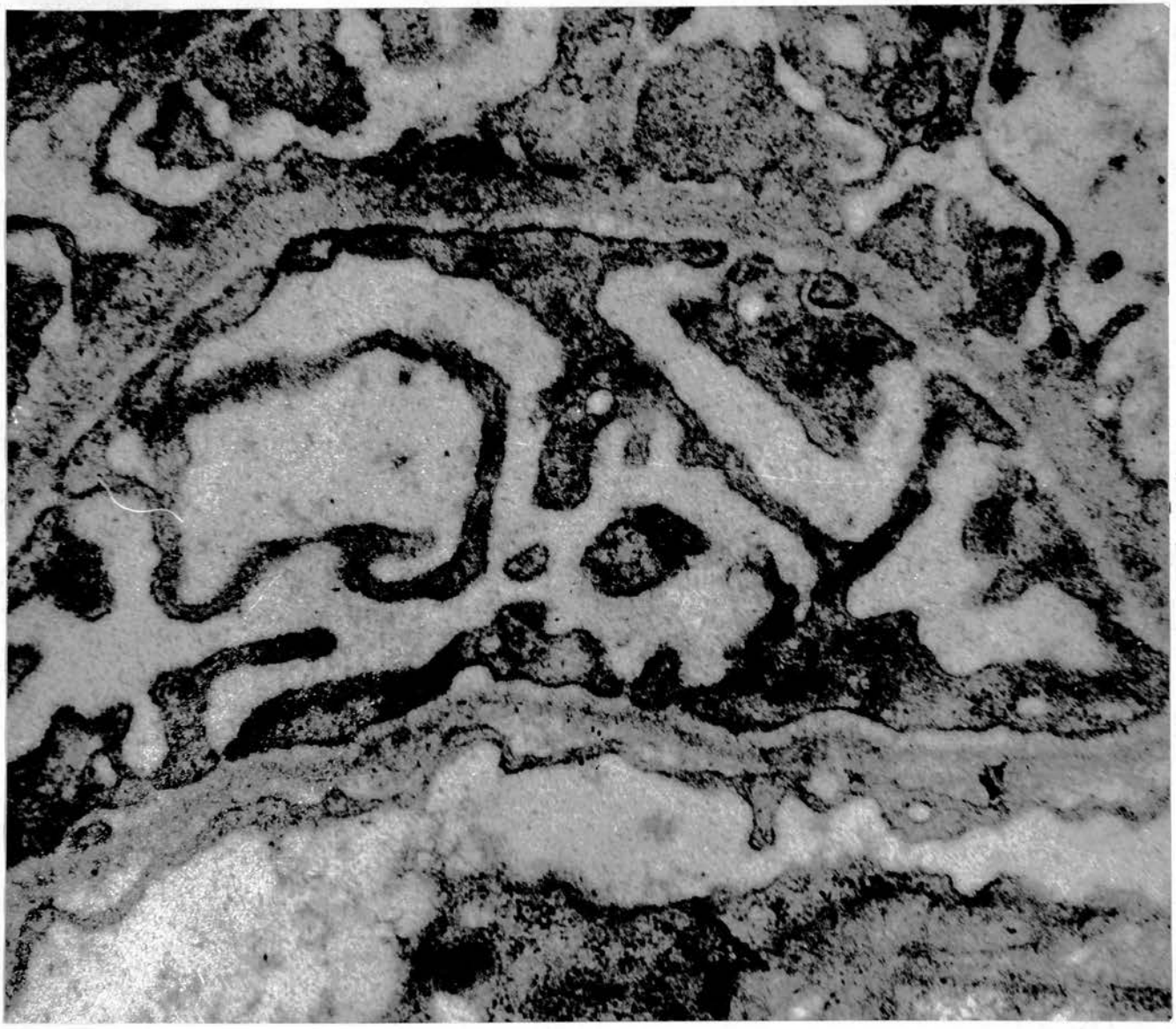


PLATE 21

Cell membrane of a visceral epithelial cell in the glomerulus, showing active budding of virus particles. This specimen, and all subsequent specimens described in this section, are from osteopetrotic birds, unless otherwise stated.

PLATE 21

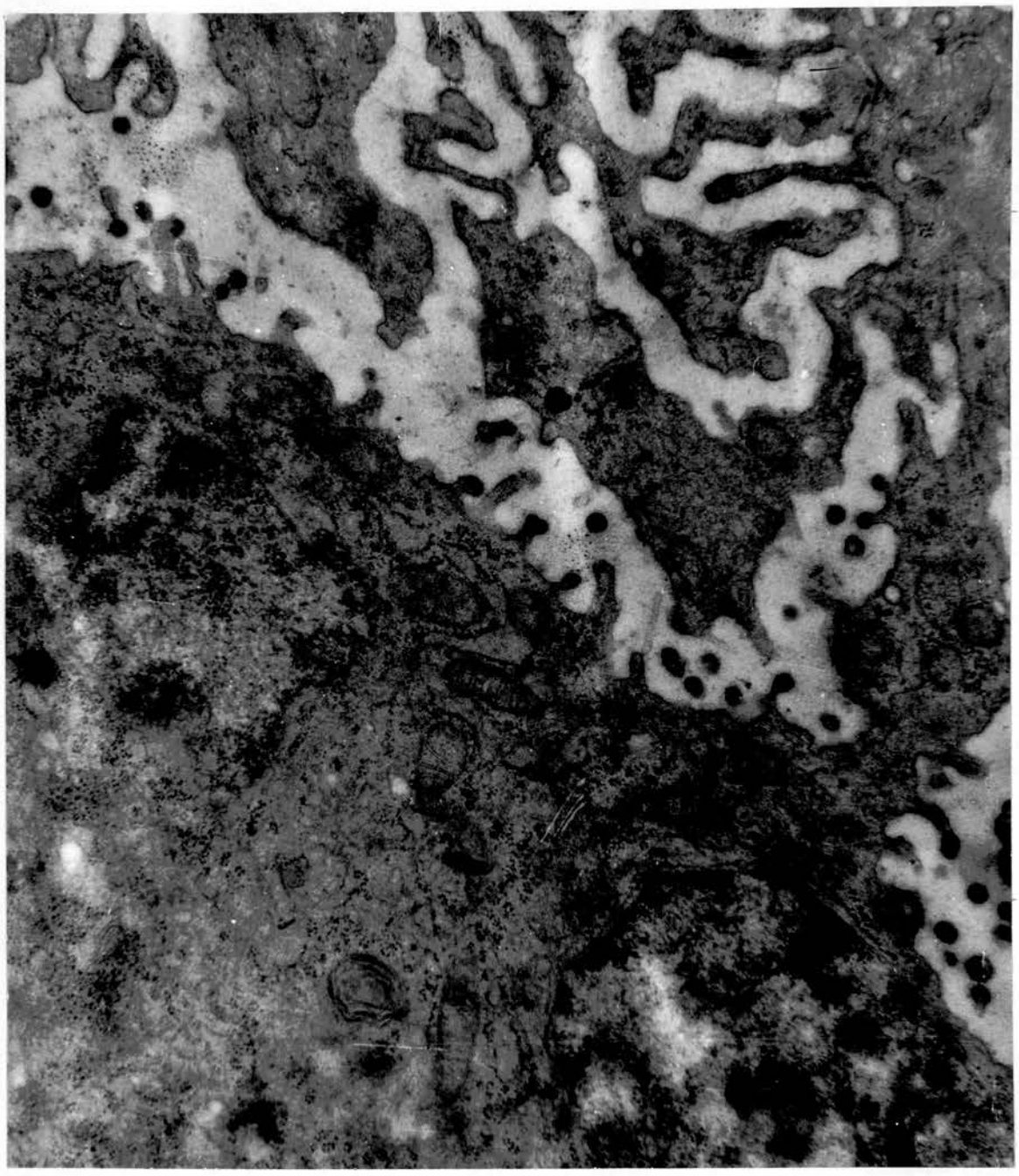


PLATE 22

Part of the glomerular region of an infected kidney nephron. The virus particles are all in the urinary space and the cells visible are visceral epithelial cells.

PLATE 22

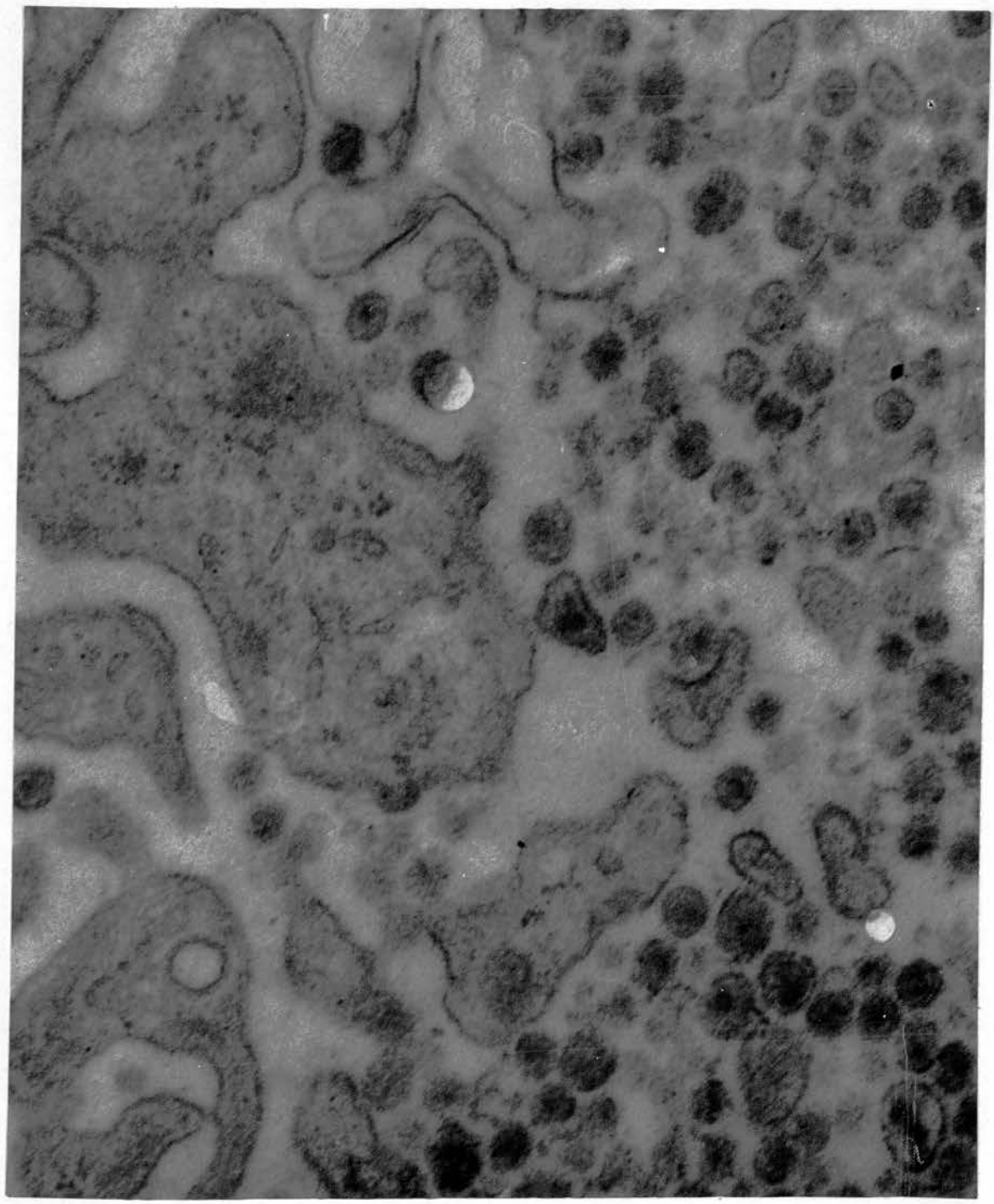


PLATE 23

Part of the brush border of a proximal convoluted tubule cell. Just below the crypts at the bases of the microvillii small pinocytotic vesicles can be seen.

PLATE 23

PLATE 24

Transverse section of a nephron in the distal region of an infected kidney. Virus particles can be seen in the inter-cellular spaces.

PLATE 24

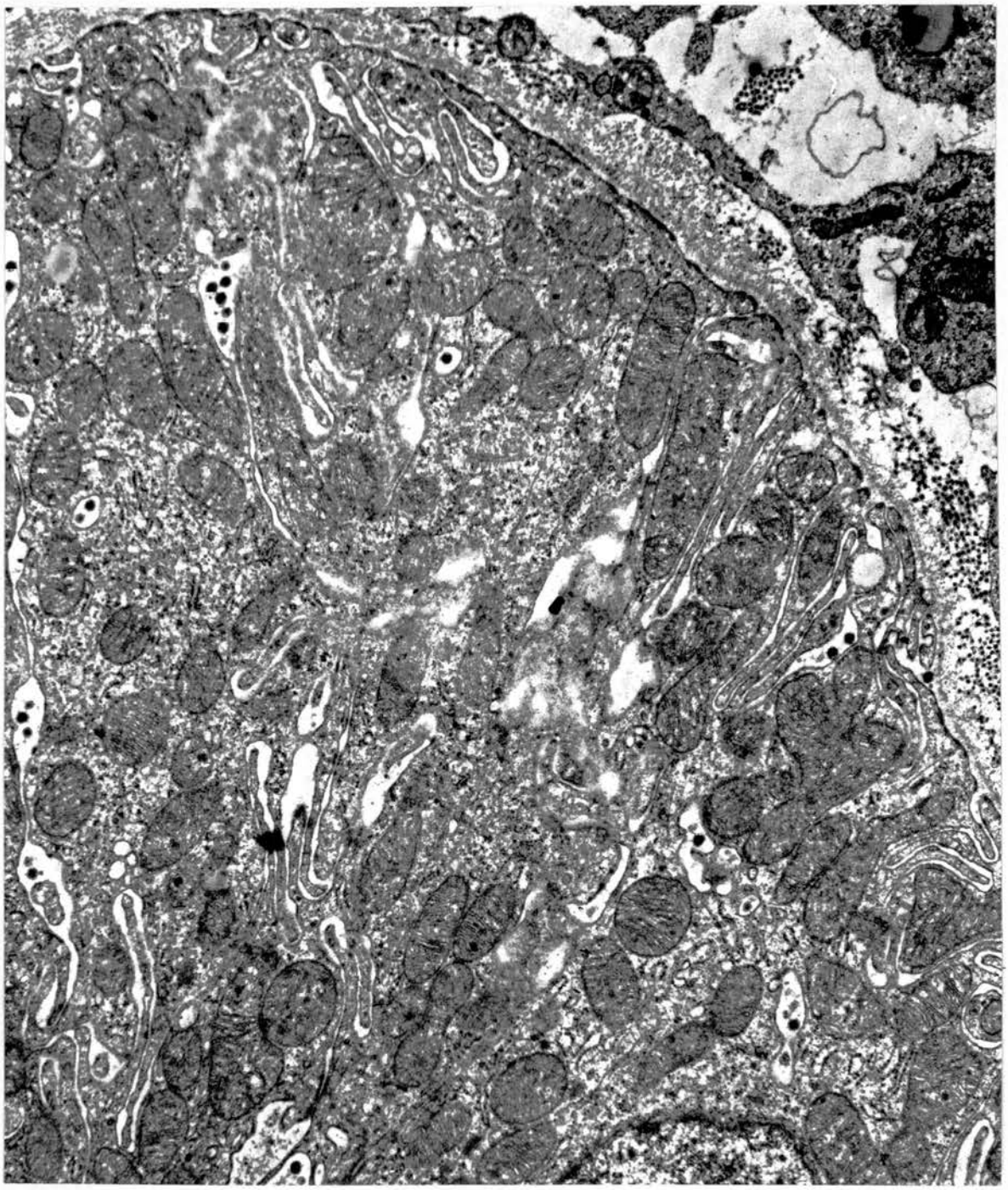


PLATE 25

Part of a distal convoluted tubule near to the basement membrane (top right). This shows the typical distribution of virus particles in this region amongst the many cell membrane invaginations.

PLATE 25

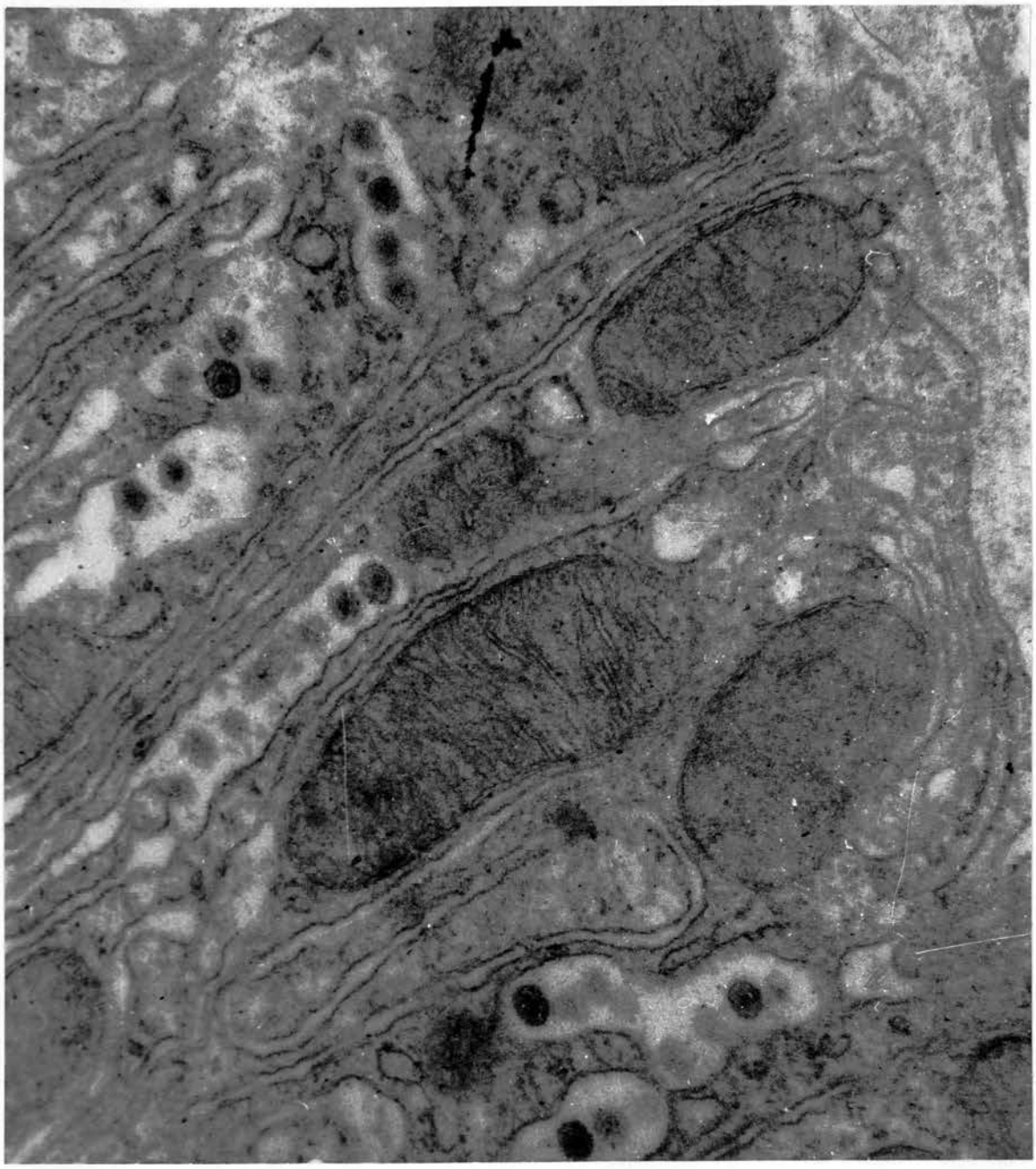


PLATE 26

Parts of two different cell types, from two adjacent nephrons can be seen in this plate. The cell on the left is from the distal region and shows the complicated infoldings typical of this region. The cell on the right is from the proximal region and shows the arrangement of the cell membrane typical of this region. The two, thin basement membranes can be seen quite clearly in this micrograph.

The white circle (centre left) is a hole in the formvar support film.

PLATE 26

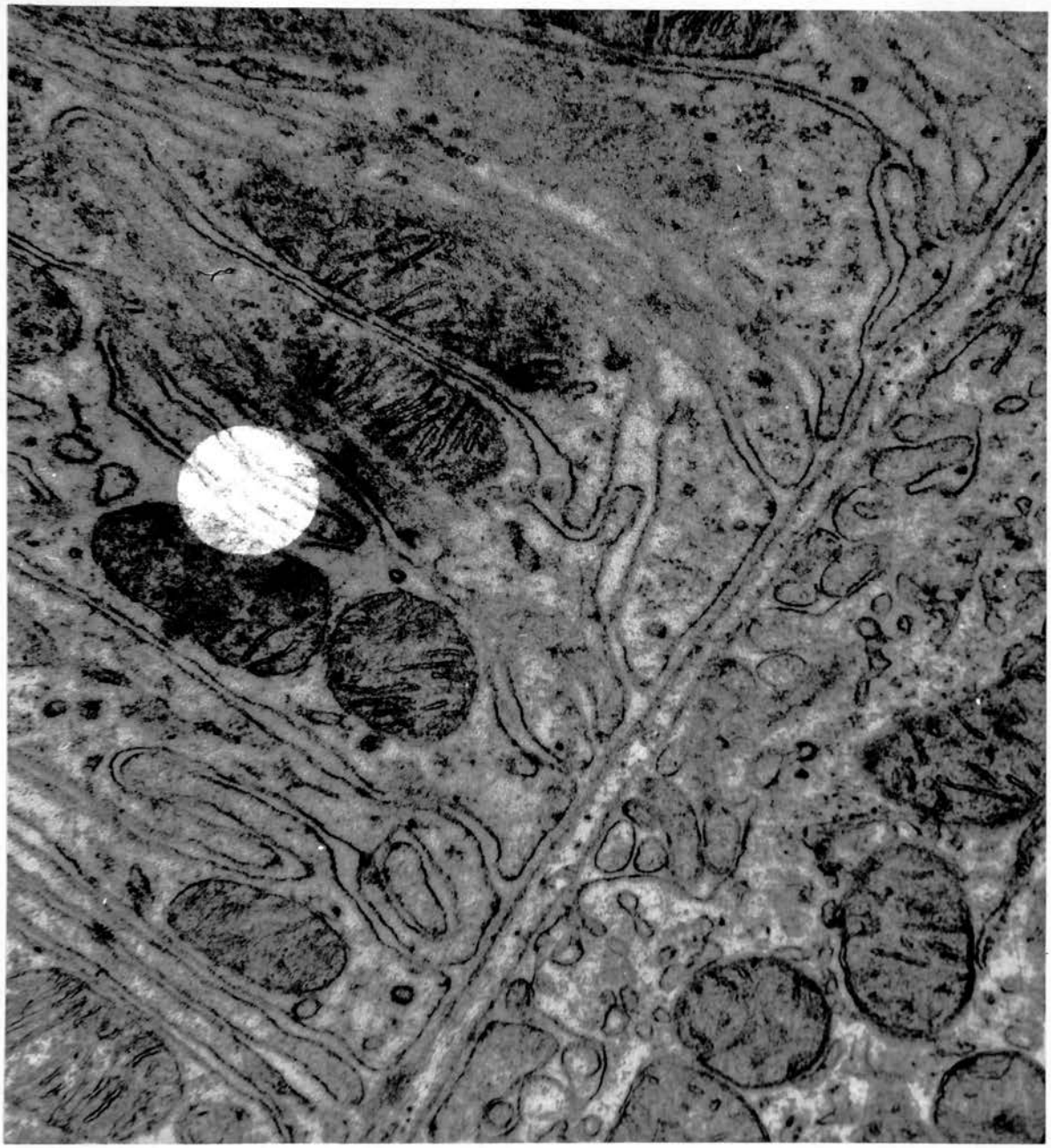


PLATE 27

Virus particles in the lumen of a kidney tubule. The next two plates show the different concentrations which can be found.

PLATE 27

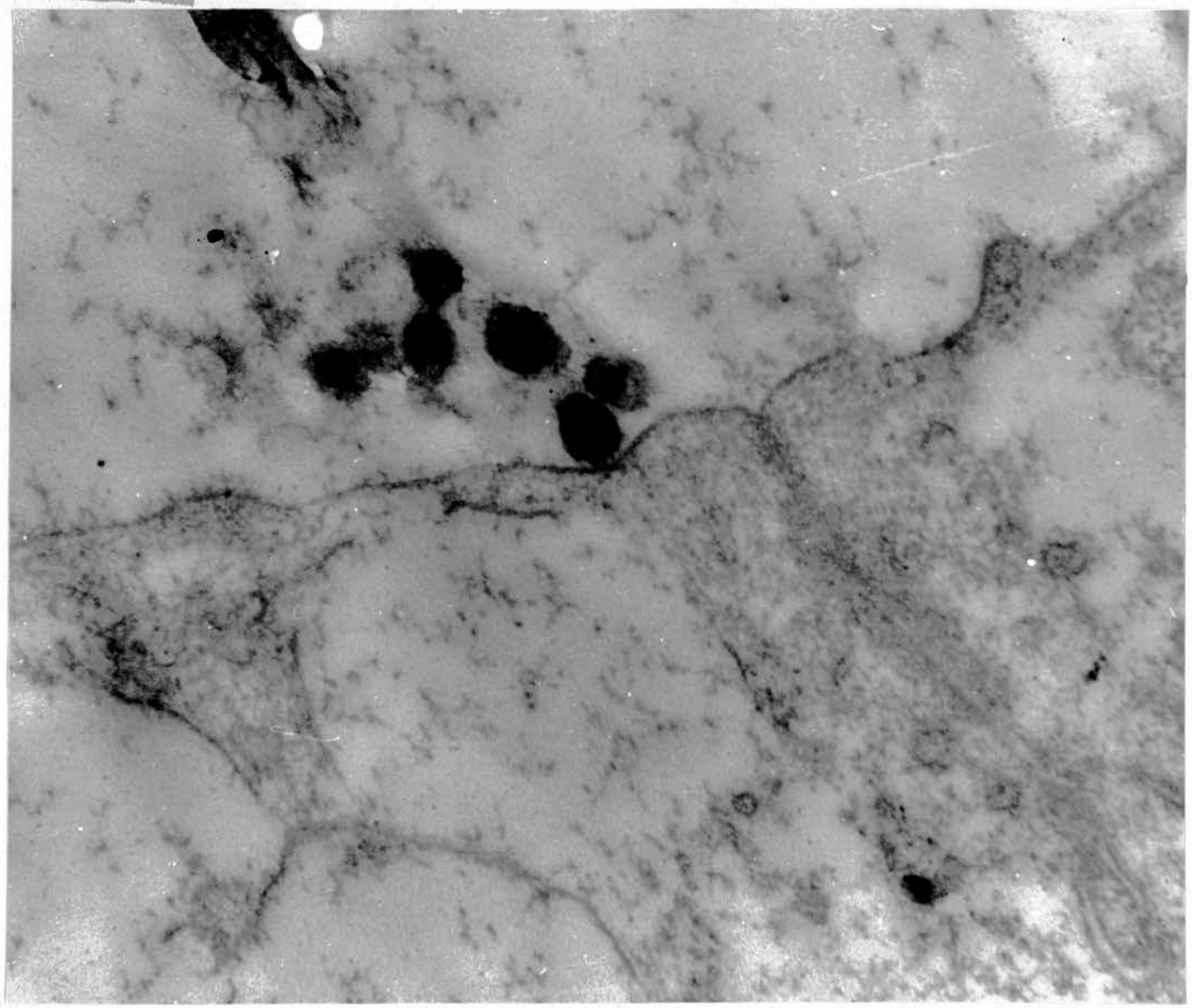


PLATE 28

Typical groups of virus particles: a bud can be seen forming from the cell membrane (bottom centre).

PLATE 28

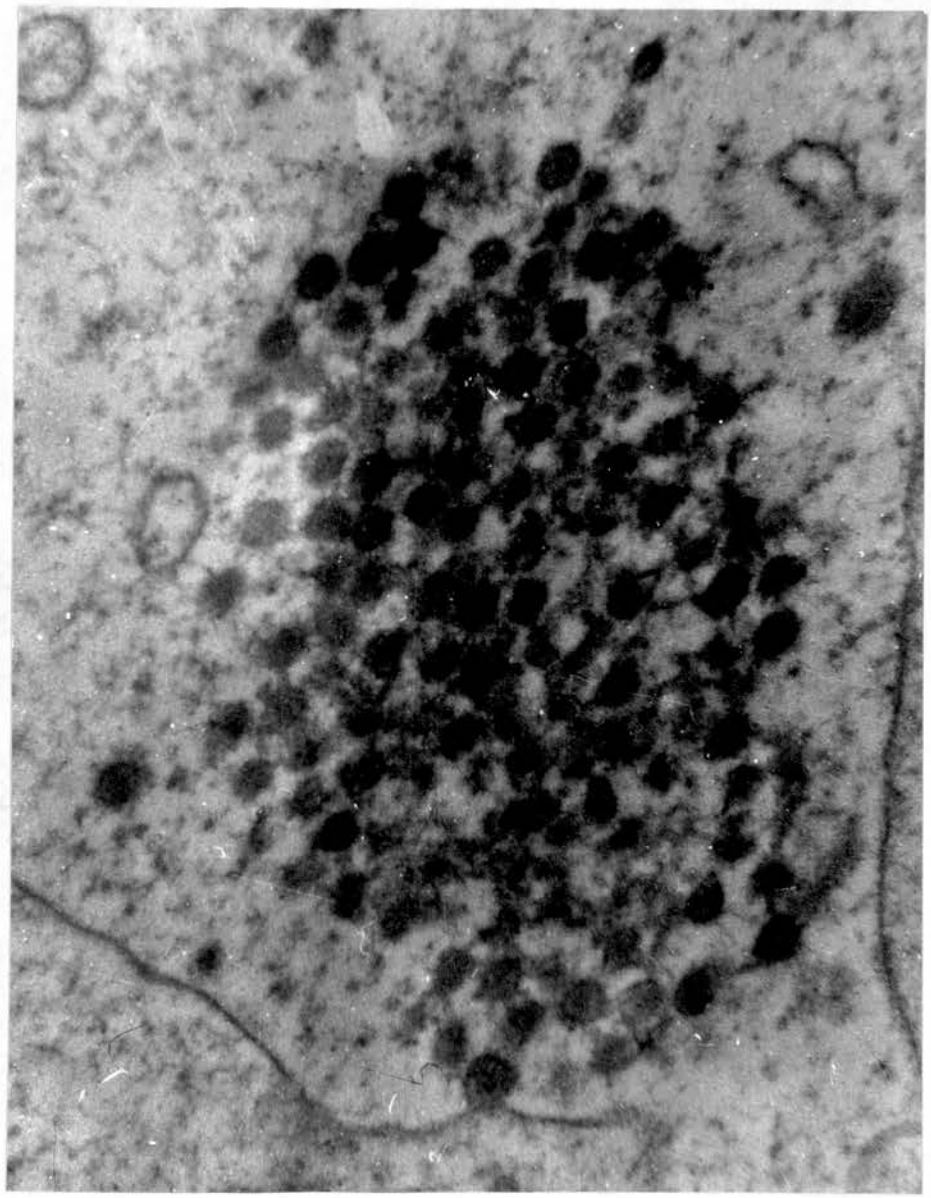


PLATE 29

A very high concentration of virus particles in the lumen. This was only very occasionally found.

PLATE 29

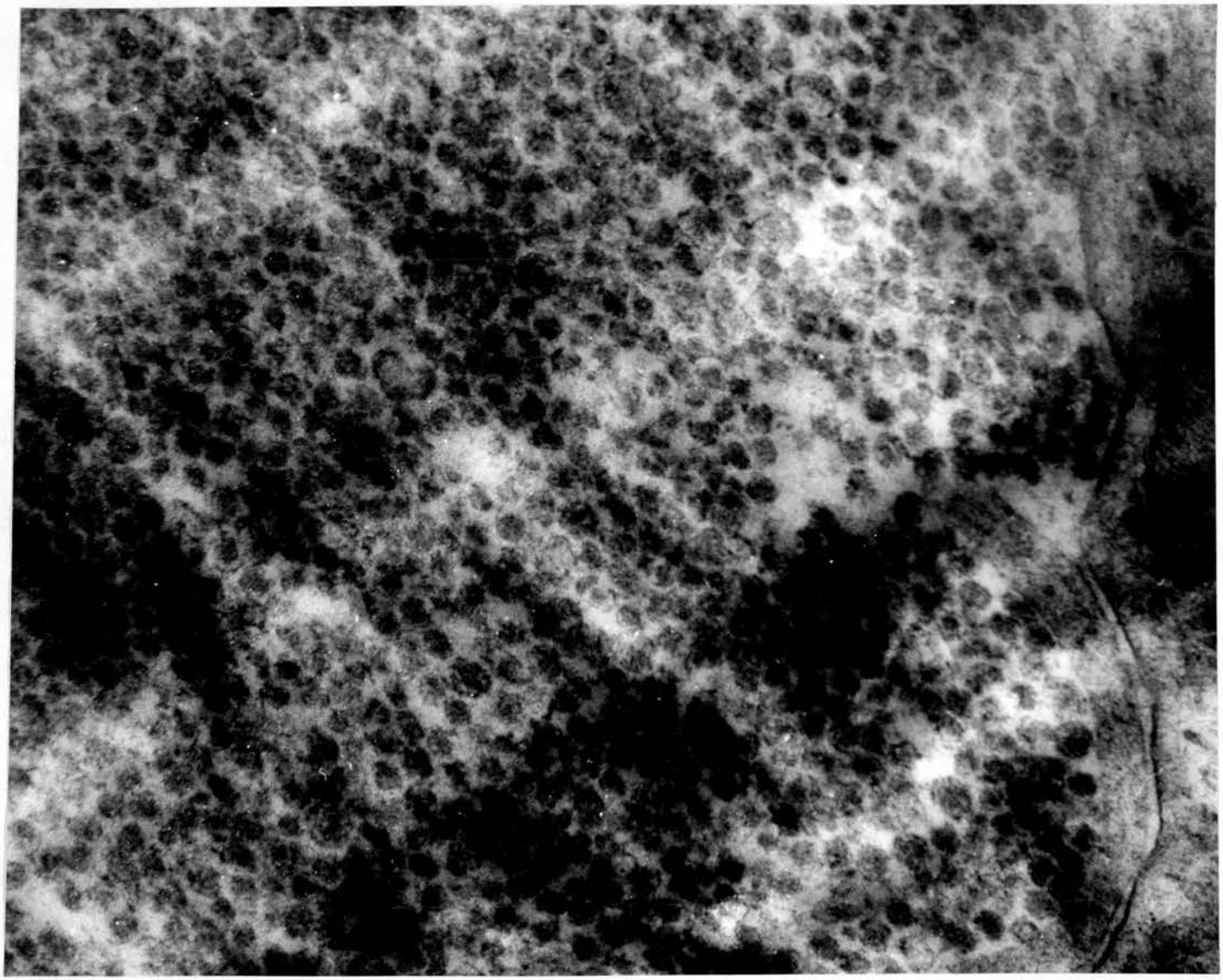


PLATE 30

This plate shows a virus particle near the basement membrane of the proximal region of a nephron. The small distance between the basement membrane and the lumen of the adjacent blood capilary is apparent. The cell at the top of this plate is an erythrocyte.

PLATE 30

PLATE 31

Virus particles can be seen near to an erythrocyte in the lumen of a blood vessel. The blood vessel has kidney cells on each side.

PLATE 31

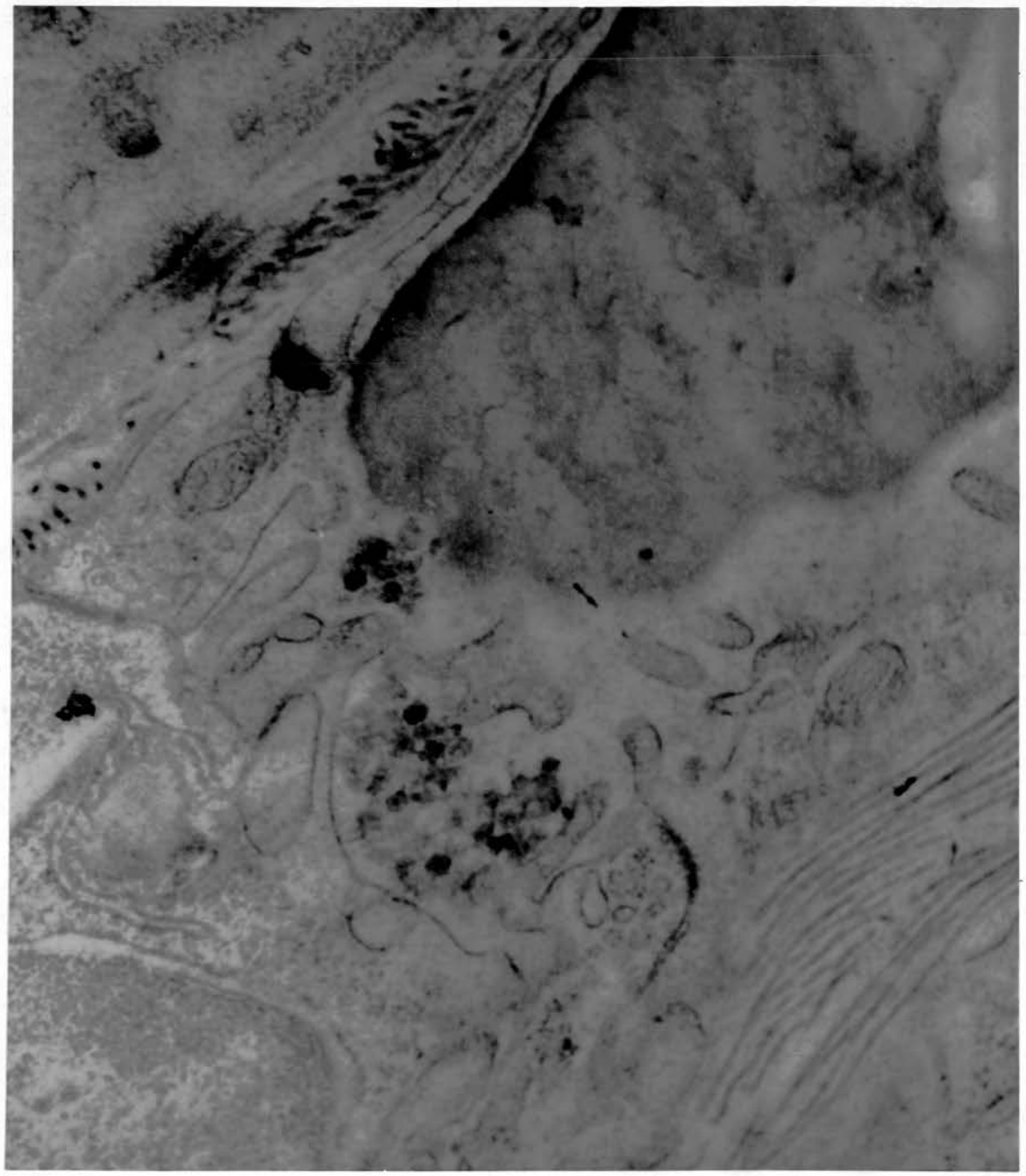


PLATE 32

Part of an osteopetrosis infected C.A.M. This micrograph has been included to show how osteopetrosis virus particles appear to have an affinity for blood vessels. The virus particles in the centre of this plate appear to be very close to the endothelial cell of the capillary.

PLATE 32

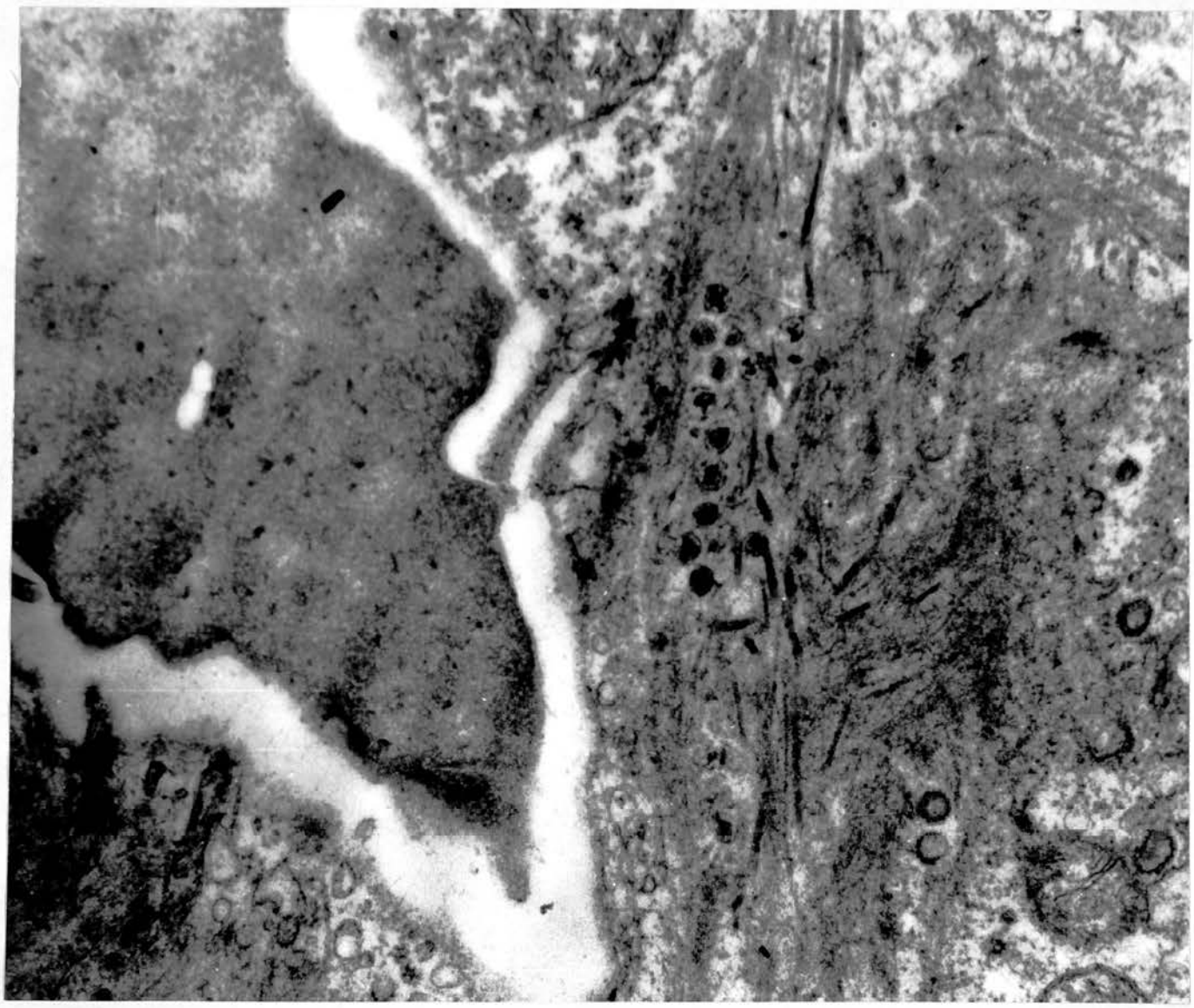


PLATE 33

Two virus particles very close to the endothelial cell of a blood capillary in the distal region of a nephron. The particles are in the region of the basement membrane although this structure is not very clear. The cell on the left is an erythrocyte.

PLATE 33

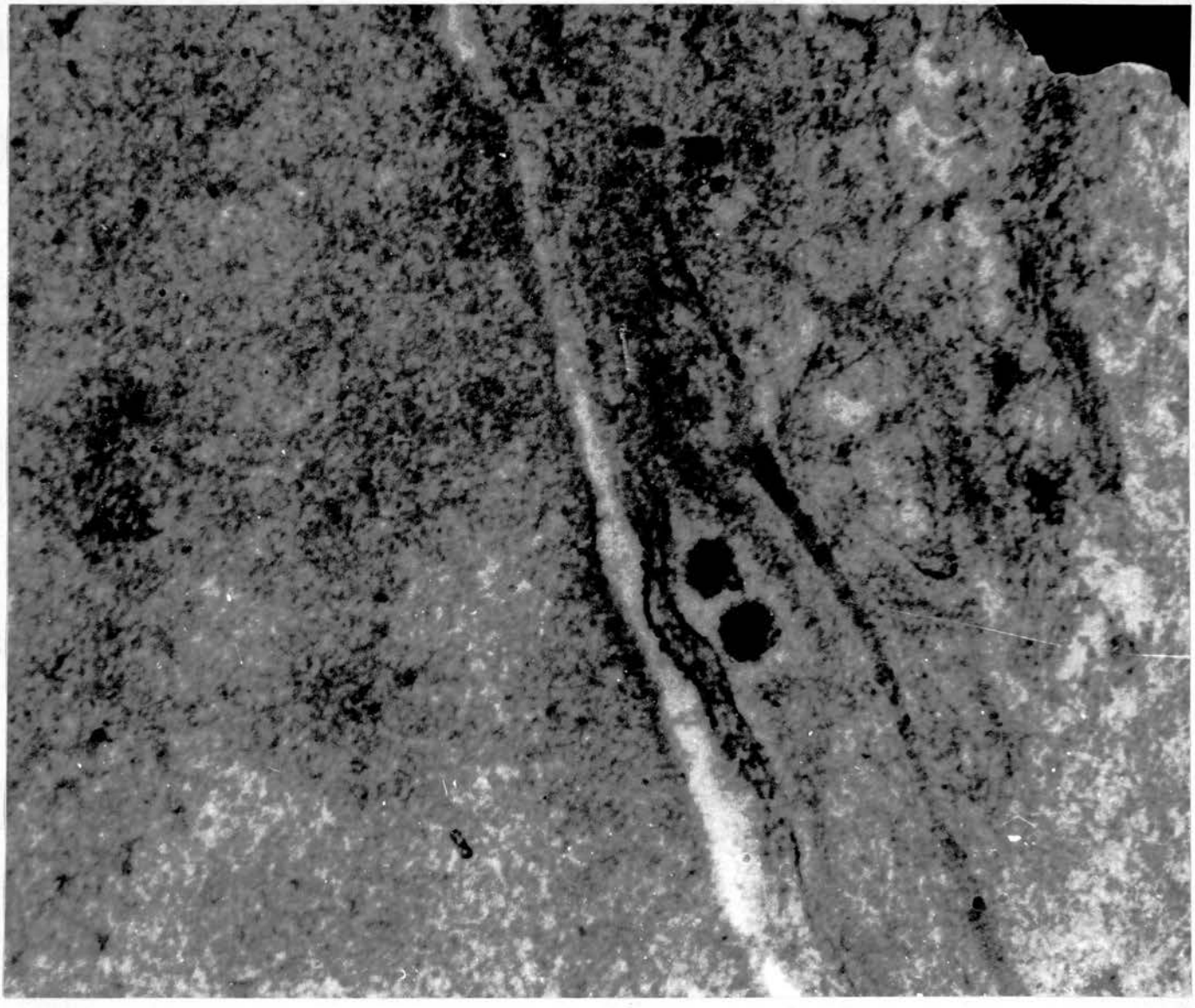


PLATE 34

Cells of the distal region of the nephron showing cilia protruding into the lumen of the tubule.

PLATE 35

Transverse section of the cilia showing the typical 9 + 2 fibre structure.

PLATE 34

PLATE 35

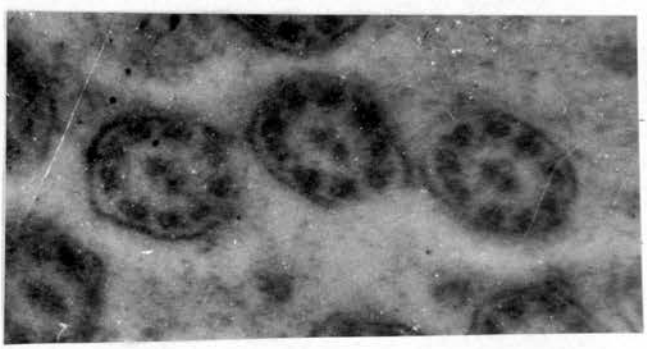
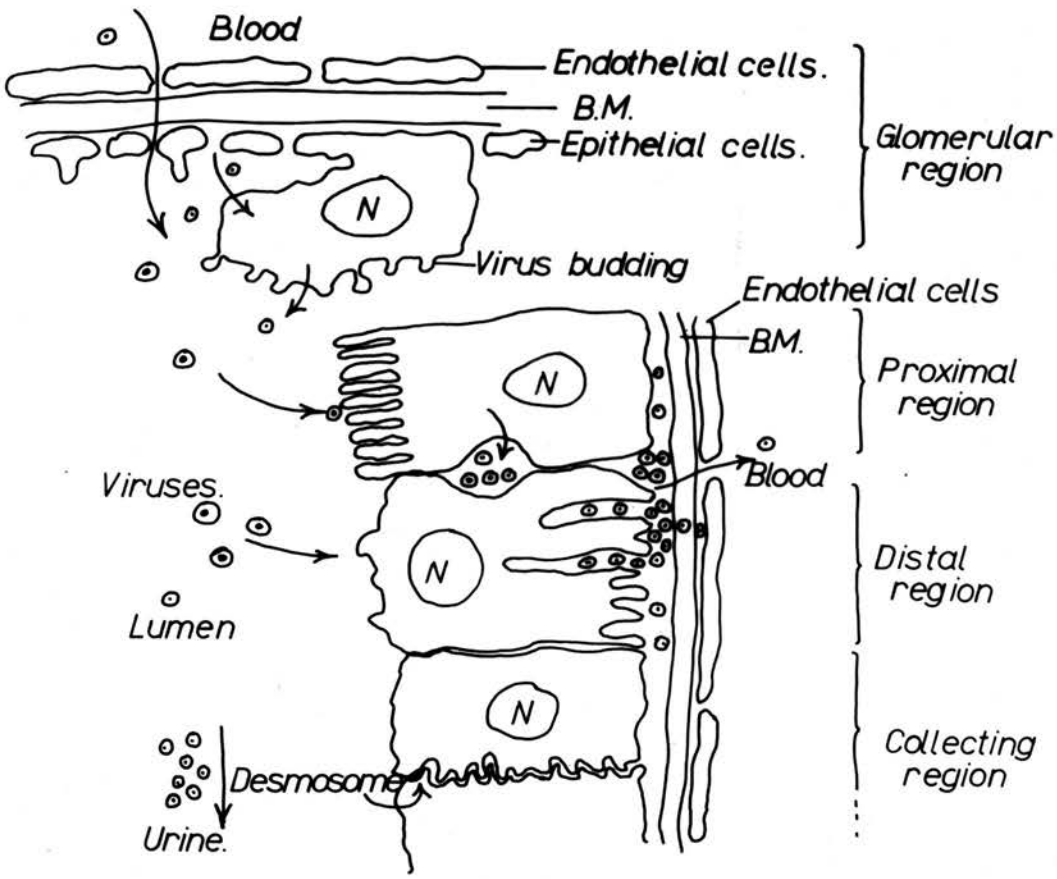


DIAGRAM III

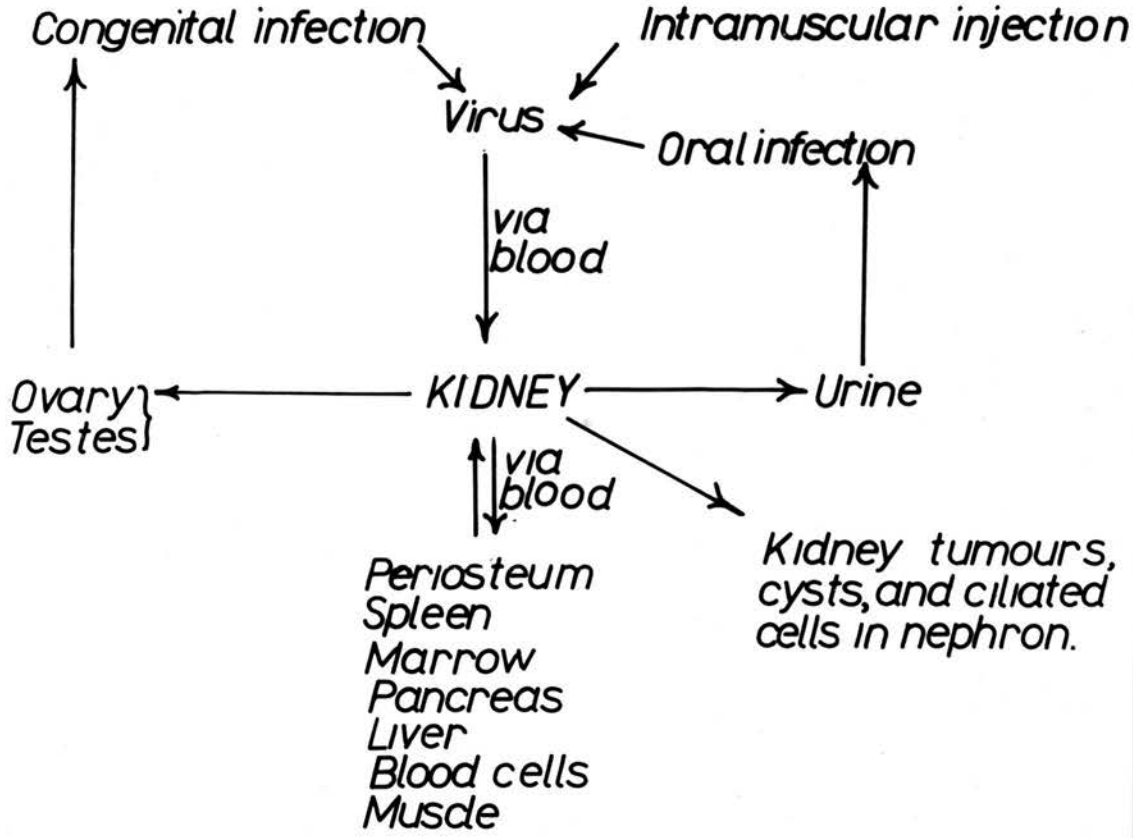
Osteopetrosis virus infection of the nephron.



N-Nucleus.
B.M:- Basement membrane.

Diagram IV

Life cycle of the Osteopetrosis virus.



SECTION VI

STUDIES WITH TUMOURS ASSOCIATED WITH OSTEOPETROSIS

P.R.C. 18

INTRODUCTION

Transplantable tumours occasionally arose in birds suffering from osteopetrosis, and although all of the tumours examined will not be described, one which proved to be of particular interest is reported in detail. This tumour, which was designated P.R.C. 18, was first transplanted from the wing of a bird that showed all the clinical signs of osteopetrosis. The bird was one in a group injected with whole blood from another florid case of osteopetrosis twelve weeks earlier, in order to passage osteopetrosis routinely. A detailed examination was carried out on P.R.C. 18, since, on serial transplantation, tumour development was found to be associated with rapid development of osteopetrosis. It was the aim of this work to elucidate the nature of this association.

An excellent review of the possible implications of a virus found to be present in a tumour can be found in a leading article of the British Medical Journal, (Andrewes, 1964). In this, the author emphasises that many viruses appear to show a preference for tumour cells, so that the mere presence of a virus in a tumour is not sufficient to show a causal relationship.

It seemed probable that the osteopetrotic relationship with P.R.C. 18 was of this 'passenger' type. Consequently,

a study of the effects of transmitting cell-free extracts to day-old chicks, known to be free from leucosis viruses and osteopetrosis, would indicate the character of any viruses present. Experiments to determine their effects were undertaken.

MATERIALS AND METHODS

Specimens of P.R.C. 18, kidneys from birds infected with P.R.C. 18, or with cell-free extracts of P.R.C. 18, were examined, using the light and electron microscopes. Specimens of spleen, pancreas, periosteum and marrow in infected birds were also examined. Fixation and preparative techniques were the same as those described in sections I and II of this work.

The tumour was passaged routinely, as cell suspensions in 0.9% saline. Cell-free extracts of the tumour were prepared by grinding up pieces of tissue in distilled water, using a glass rod in a large test tube. The resulting suspension was then centrifuged at 1,500g., to remove the cells. 0.2 ml. aliquots of this suspension were used to infect day-old chicks intramuscularly

RESULTS

Passage experiments. A summary of these results can be found in table 12, page155. It can be seen from this table that a number of birds inoculated with the cellular fraction of this tumour developed osteopetrosis, as well as a large tumour. The age at which these birds showed clinically distinguishable features of osteopetrosis was as low as six weeks

in some cases. In the experiments where cell-free extracts of P.R.C. 18 were used as inocula, all the birds developed osteopetrosis in its normal form, without P.R.C. 18 tumours. In table 12, page 155, only eight birds are recorded as having osteopetrosis lesions as well as P.R.C. 18 tumours. However, since the birds developing P.R.C. 18 tumours were usually killed when they were between three and eight weeks old, even a low incidence of osteopetrosis is significant. In order to examine the intimate relationship of the osteopetrosis virus and the P.R.C. 18 cells, a detailed examination of the tumour was carried out.

Microscopical Examination. The light microscope examination revealed a similarity between P.R.C. 18 and the small tumours often found in the breast and leg muscles of birds suffering from osteopetrosis. Histologically, the tumour consisted of a disorientated array of muscle cell precursors, which, with difficulty, would occasionally be shown to contain typical muscle cell striation. These cells were interspersed with considerable numbers of connective tissue cells, and the periphery of the tumour merged indistinctly with normal muscle cells. Examination at higher magnifications with the electron microscope confirmed these observations, but the myofibrils of the muscle cell precursors were seen rather more easily. The cells of the tumour, thought to be connective tissue cells, were found to have cytoplasm containing a bare minimum of the normal ultrastructural components (plate 36, page 159). There were very few mitochondria, a sparse endoplasmic reticulum, few

free ribosomes and not very many cellular inclusion bodies. Plasma cells were frequently found throughout the tumour tissue.

Virus particles were observed in both intercellular and intracellular locations. However, the particles found in the two locations differed considerably in their morphology. One particle was an osteopetrosis-like particle, and the other a much smaller particle, whose fine structure was not clearly seen.

Osteopetrosis-like particle (plate 37, page 160). These particles had a similar structure to osteopetrosis particles. They consisted of a central, dense nucleoid, which was approximately 700Å in diameter, surrounded by two slightly eliptical membranes, giving an overall structure of 1,100Å across one diameter, and 950Å across the other. The particles were invariably intercellular, and were found in small groups of between two and twenty (occasionally singly), immediately adjacent to cell membranes. The cells which were most frequently associated with these particles did not have any myofibrilae, and were probably of connective tissue origin. Budding was occasionally observed from these cells, suggesting replication similar to that illustrated in plates 9 - 12, pages 42 - 45. Particles were not seen concentrated near plasma cells.

Cytoplasmic particle (plate 41, page 164). There is no concrete evidence that this particle was, in fact, a virus, and it may have been an unusual cytoplasmic component. It does, however, appear in several of the tumour cell lines maintained

at the Poultry Research Centre, and its distribution and numbers, as well as its size, are consistent with known cytoplasmic commensal viruses. The particle was always found in the cytoplasm, and usually appeared in groups of about forty, in sections of tissue. It was approximately 300Å in diameter, and seemed to consist of a doughnut-shaped structure. These particles were also found in R.P.L. 12, P.R.C. 13, and P.R.C. 18, and plate 41 shows these particles in R.P.L. 12 cells. Details of the ultrastructure were not adequately studied, since the resolution of the Phillips E.M. 100, used during this work, was only about 25Å.

Infected kidney. The kidneys of birds infected with this tumour quickly became infected with an osteopetrosis-like virus particle. Within a week of infection with P.R.C. 18 tumour cells, the kidneys showed considerable quantities of virus particles and budding was observed from cell membranes of the epithelial cells in the glomerulus.

DISCUSSION

From these results, it can be concluded that P.R.C. 18 probably has two viruses replicating in its cells. Whereas there is no evidence that either of these virus particles is responsible for the continued propagation of the tumour, the fact that osteopetrosis results from passaging cell-free extracts suggests that the larger particle described was, in fact, the virus responsible for osteopetrosis. Furthermore, this particle is actively replicating in the cells of the

tumour, and is not being continuously passaged in the blood contained in the tumour material. Although there is no definite evidence to suggest that this virus was responsible for the original P.R.C. 18 tumour, equally, there is no evidence to the contrary.

The kidney infection is probably due to the fast growing P.R.C. 18 tumour cells liberating large numbers of viruses into the blood. Injection of P.R.C. 18 cells, contaminated with osteopetrosis, would produce a richer source of virus at an earlier age than would an injection of free virus. Consequantly, the organ or tissue with the greatest susceptibility to the virus might be expected to show demonstrable virus quite soon after the infection of the bird with P.R.C. 18. Replication of virus particles in the kidney after about one week is not unexpected, and a virus infection of the periosteum after six weeks in consistent with a faster rate of development of the disease.

This virus is, then, behaving like a typical passenger virus.

Since osteopetrosis was shown to behave like a passenger in P.R.C. 18, a comparison was drawn with the reported association of osteopetrosis, lymphoid leucosis, and other viral-induced conditions, with the lymphoid tumour R.P.L. 12, first described by Burmester and his associates (1946).

HISTORY OF THE TUMOUR DESIGNATED R.P.L. 12 BY BURMESTER

Olson (1941) isolated a transplantable lymphoid tumour from the distal end of the femur of a Rhode Island Red x Barred Plymouth Rock chicken. Olson described the bird as having a large number of monocytic tumours. Campbell (1963b) considers this tumour to be a monocytic type, on the basis of its histology, and this would explain Olson's observation that the original birds had abnormally large numbers of monocytes in the blood. Attempts by Olson at passaging this tumour were only successful as cellular suspensions, and cell-free extracts of tumour material, together with cell-free blood from the original birds, failed to produce any abnormalities after injections into young birds, up to 96 days later. The only pathological condition Olson reported in his birds was fowl paralysis, with which 15 out of his 443 birds were infected. Burmester et al (1946) obtained this tumour, and gave it the designation R.P.L. 12. These workers showed that cell-free filtrates of blood and tumour extracts produced osteopetrosis and lymphoid leucosis after injection into day-old chicks. However, they also reported that the tumour had been previously passaged 50 to 200 times in their flock of birds. Nelson et al (1946), and Burmester and Denington (1947), have all described the high incidence of visceral, neural, and occular lymphomatosis in the flock Burmester has worked with. The existence of erythrogranuloblastosis and osteopetrosis have also been recorded in

'a few' birds of the same flock, (Burmester and Denington, 1947). Consequently, in the light of present knowledge of passenger viruses, it is not surprising that the study of R.P.L. 12, grown in birds with a relatively high background incidence of the diseases described, revealed a widening spectrum of pathological conditions. There seems every justification for Nelson et al (1946) to comment at the end of their paper that "the use of inbred lines of chickens, free from all known diseases, will lead to better understanding of avian tumours".

The association of osteopetrosis with R.P.L. 12 is again described by Burmester and Cottral (1947), and then Burmester (1947a) isolated osteopetrosis from two other tumours, R.P.L. 18, an ovarian tumour, and R.P.L. 21, a lymphoid tumour. The significant point in the case of R.P.L. 21 is that osteopetrosis virus was not evident in cell-free extracts of tumour material for the first six passages, but was quite common after the sixth passage. Burmester (1947b) does mention the possibility of there being more than one 'agent' present in his cell-free material, but argues that the dose of virus is important in determining the latent period, and perhaps the condition itself, (Gottschalk, 1946; Bryan, 1946). Burmester and Gentry (1954) have suggested that the agent responsible for osteopetrosis differs from that responsible for lymphomatosis since the former is not transmissible on contact. However, the concept of a multipotency for the causal agent of the leucosis group of conditions, osteopetrosis and lymphoid leucosis, is presented by Burmester et al,

(1959). Multipotency was considered, since this group of workers found that Beard's strain-A myeloblastosis produced myeloblastosis, lymphoid leucosis, carcinomas and osteopetrosis and Beard's strain-R erythroleucosis produced erythroleucosis and lymphoid leucosis: previously, both these strains had been considered 'pure', since they had been maintained in tissue culture, thus avoiding contamination from outside sources.

Further evidence for the concept of a multipotent virus with an oncogenic spectrum is advanced by Burmester et al (1960a), who showed that R.P.L. 12 material could also produce erythroblastosis, as well as lymphoid leucosis and osteopetrosis. Burmester et al (1960b) demonstrated another virus from R.P.L. 12, which they called Gallus-adeno-like (G.A.L.) virus. However, this virus was shown antigenically, and from its cytopathic behaviour in tissue culture, to be unrelated to the leucosis group of viruses (Stoker, 1959).

Finally, Burmester and Walter (1961) claim that extracts of R.S.V. from Rous tumours have produced visceral lymphomatosis and erythroblastosis, which again seems to point to the probable presence of contaminating viruses.

An analysis of this data reveals:

1. when first described by Olson, the R.P.L. 12 tumour could not transmit any pathological condition by cell-free extracts:

2. after 50 to 200 passages (in 1946), lymphoid leucosis and osteopetrosis could be extracted from the tumour and blood of birds infected with ~~cell-free extracts of~~ R.P.L. 12:

3. erythroblastosis was produced in birds injected with

cell-free extracts of R.P.L. 12:

4. the evidence for the multipotency of a single agent producing erythroleucosis, myeloid leucosis, lymphoid leucosis, Rous sarcoma, as well as osteopetrosis, has been based on experiments carried out in flocks of birds with a high background incidence of these diseases.

The ability of tumour cells to become contaminated with passenger viruses has been demonstrated with Birkett's lymphoma and the P.R.C. 18 tumour described, and many other examples. As a result of these observations, and from the examination of the literature relating to R.P.L. 12 and osteopetrosis, it seems reasonable to suggest that the association of these two conditions is due to a passenger virus.

The work in this thesis has shown that the osteopetrosis virus has a rather complex life cycle, but that birds injected with the virus become infected, and actively produce new virus after about three weeks. Not all birds develop bone lesions, but almost all produce new virus in the kidney. Consequently, birds can become viraemic with respect to osteopetrosis virus, but may not necessarily show obvious signs of the disease. If this pattern of viral development is the same as that of osteopetrosis in the United States, then it is quite probable that R.P.L. 12 could have been routinely passaged into birds which were viraemic, without the researcher being aware of the infection. If osteopetrosis could then behave like the reo, or herpes simplex viruses associated with Birkett's lymphoma, or the osteopetrosis virus in P.R.C. 18, then contamination of the R.P.L. tumour might result. Since the flock maintained

at the Poultry Research Centre in Edinburgh is considered to be leucosis free, experiments were undertaken to determine the nature of the R.P.L. 12 tumour and its relationship with osteopetrosis. Firstly, an examination was undertaken of the original R.P.L. 12 tumour maintained at the Poultry Research Centre, in order to determine the oncogenicity of cell-free extracts. A microscopical survey of the tumour was also carried out.

MATERIALS AND METHODS

The R.P.L. 12 tumour was obtained in 1960 from Dr. R.J.C. Harris, who had passaged it several times in chickens, after previously obtaining a transplant from Dr. Burmester. R.P.L. 12 has been routinely passaged by cellular transplants in the flock of Brown Leghorn chickens at the Poultry Research Centre ever since. Cell-free extracts from the tumour and from the blood of birds infected with the tumour were prepared and passaged in the same way as that already described for P.R.C. 18 material. The tumour was examined by the usual light and electron microscope techniques. Specimens of the kidney, spleen, pancreas and liver of R.P.L. 12 infected birds and birds injected with cell-free extracts were also examined histologically.

RESULTS

Passage experiments. R.P.L. 12 has never been associated with any leucotic condition, or with osteopetrosis, during the four years it has been passaged by cellular suspensions at the Poultry Research Centre.

Cell-free extracts of tumour tissue produced no apparent pathological response in the treated chickens, even though some birds were kept for almost a year before being killed and examined.

Microscopic examination. The appearance of R.P.L. 12, as transmitted in this laboratory, is not altogether identical to Burmester's (1946) or Olson's (1941) descriptions. On the contrary, the cells appear to be monocytic in type, rather than lymphoid (plate 38, page 161).

When living cells were examined, they were seen to be actively phagocytic, engulfing small particles of carmine, which is not a characteristic of lymphoid cells (Campbell, 1963). Similar conclusions were reached by Darcel and Negroni (1954).

Careful examination of tissue sections with the electron microscope did not reveal any intercellular virus particles of the leucosis type. However, two small, distinctly different particles were seen.

The first of these, which was also found in P.R.C. 18 tissue, was of the doughnut type. This particle appeared to be identical with that already described on page 136 , and its significance has been discussed.

The second particle was found in considerable quantities and was obviously cytopathogenic. Replication of this particle appeared to begin in the nucleus, with subsequent migration into the cytoplasm, resulting in the latter gradually filling up with virus. In the final stages, infected cells

were packed with virus particles, (plates 39, 40, pages 162, 163). The cells then apparently burst and, presumably, released particles, to repeat the cycle in other cells.

DISCUSSION

The cytopathic virus found in R.P.L. 12 tumour cells was probably that described by MacPherson et al (1961), and called Gallus-adeno-like (G.A.L.) virus. They describe this virus as having an average diameter of 975Å and a polygonal, usually hexagonal, profile. The reason for this rather clumsy name is that the virus shares morphological features with adeno-type 5 virus, and also herpes simplex and polyoma. This virus has been reported as being cytocidal in tissue culture, (Stoker, 1959).

For some time, this particle was claimed to be responsible for lymphomatosis (Fontes et al, 1958; Sharpless et al, 1958), but Stoker (1959) was the first to point out that a cytocidal virus of this type behaved very differently in culture to the R.S.V. When R.S.V. replicates in chick fibroblast cultures, it produces 'mounds' of cells (Temin and Rubin, 1958) which indicates oncogenicity, whereas G.A.L. virus kills cells. Subsequently, Burmester et al (1960) retracted their statement that G.A.L. virus was responsible for lymphomatosis, on the grounds of antigenic differences between G.A.L. and the leucosis viruses.

CONTAMINATION EXPERIMENTS

INTRODUCTION

The history of G.A.L. virus is another example of the danger of relating a tumour with a viral agent on insufficient evidence. Furthermore, the survey reveals that the R.P.L. 12 tumour, passaged in this laboratory, does not contain leucosis-type viruses, and that it cannot be passaged by a cell-free preparation. The significance of this will be discussed at the end of this section. Knowing this tumour to be free from leucosis-type viruses, detectable by the techniques available, it was attempted to show that R.P.L. 12, like P.R.C. 18, could act as a source of virus, without itself having a viral aetiology. In other words, it was hoped to infect R.P.L. 12 with a virus which would, in fact, be a 'passenger', but would behave in the same way as viruses described by Burmester.

MATERIALS AND METHODS

Contamination of R.P.L. 12 with R.S.V. and osteopetrosis viruses was attempted. These two viruses were chosen since they are rather different in character, but are both mentioned by Burmester as being associated with leucosis conditions. The frequent classification of osteopetrosis as one of the leucosis group of viruses is due entirely to the R.P.L. 12 work. R.S.V. was chosen since its presence can readily be detected by titration in day-old chicks. Occasionally, R.S.V. can remain in a latent state, in birds, for several months,

(Carr, 1961), but since the strain of birds used during this work was considered R.S.V. susceptible, the possibility of latency was discounted. The procedure for contaminating R.P.L. 12 cells, in a way similar to that which might normally be expected to take place in chickens, was as follows:

1. R.P.L. 12 cells were injected into birds showing advanced osteopetrosis lesions, i.e. thick bones and anaemia.

2. R.S.V. contamination was carried out by intramuscular injection of Rous Sarcoma cells into one leg of a chicken, followed, two days later, by injection of R.P.L. 12 cells into the other. The reason for this interval between injections was to avoid the contamination of R.P.L. 12 with any Rous Sarcoma cells which may have been injected into the blood.

3. In order to contaminate R.P.L. 12 with both osteopetrosis virus and R.S.V., the R.P.L. 12 tumour, previously passaged into a bird with osteopetrosis, was treated as in (2).

After R.P.L. 12 tumours had been grown in either the osteopetrosis or the Rous infected birds, until the birds showed signs of distress, the birds were killed, and then the contaminated tumours were passaged routinely as cell suspensions. At each passage, cell-free extracts were obtained from these tumours and injected into day-old chicks. The tumours and specimens of kidney, pancreas, spleen and (in the case of a bird possibly infected with osteopetrosis) the periosteum, were examined with both light and electron microscopes, using the usual techniques. Birds possibly infected with osteopetrosis were killed after six weeks.

RESULTS

The results of the passage experiments are summarised in tables 13, 14, 15, on pages 156, 157, 158.

R.P.L. 12 grown in birds suffering from osteopetrosis. Birds which had been injected intramuscularly with cellular suspensions of R.P.L. 12, and which were kept for two to three weeks, developed only R.P.L. 12 tumours at the site of the injection.

The passage experiments, in which extracts of the R.P.L. 12 tumour grown in osteopetrotic birds were injected into day-old chicks, indicated that a large proportion of the day-old chicks eventually became infected with osteopetrosis (table 13, page 156).

None of the birds injected with cell-free extracts from the R.P.L. 12 tumour developed any other lesions, except those which might have been expected to have been associated with osteopetrosis.

R.P.L. 12 grown in birds previously injected with Rous Sarcoma cells. Cellular transplants of this tumour, like that of the osteopetrosis infected tumour, produced apparently unchanged R.P.L. 12 tumours only. Cell-free extracts, however, when injected into day-old chickens, resulted in the growth of Rous Sarcoma tumours at the site of injection. The ability to produce Rous Sarcomata appeared to be getting less in the subsequent cell passages of this tumour, and the reduction in R.S.V. titre with each passage of the R.P.L. 12 tumour was apparent in the length of time it took for the Rous Sarcomata to develop.

R.P.L. 12 first passaged in an osteopetrotic bird, and then in a Rous infected bird. This tumour behaved in a way very similar to the R.P.L. 12 tumour infected with R.S.V. alone. Once again, there was a gradual reduction in the ability of the tumour to transmit Rous Sarcomata in cell-free suspensions (table 15, pages 158).

Light and electron microscope examination of contaminated R.P.L. 12 material.

R.P.L. 12 contaminated with osteopetrosis. Light microscope examination of this tumour revealed a tumour type identical to the previously described R.P.L. 12 tumour. In a more detailed examination with the electron microscope, the presence of osteopetrosis-like particles was demonstrated. The distribution of these particles was very like that observed in P.B.C. 18 tumours; the particles were in relatively small numbers, compared to the numbers expected in osteopetrosis infected kidney, periosteum or muscle lesions, but were actively replicating in the cells, since budding was occasionally observed. Osteopetrosis-like viruses were also found in the kidneys of some of the birds infected with this tumour.

R.P.L. 12 contaminated with Rous. In a comparison with non-contaminated R.P.L. 12, the light microscope revealed no change in this tumour. Many sections of this tumour from different specimens were examined in great detail. Virus particles, other than the two cytoplasmic particles described on page 144 , were not found in this tissue. The cells were also closely examined and compared to the cell types of uncontaminated R.P.L. 12 cells.

This revealed that the contaminated tumour was identical to uncontaminated R.P.L. 12.

R.P.L. 12 contaminated with Rous, after previous osteopetrosis contamination. The morphology of this tumour was identical in all respects to R.P.L. 12 infected with osteopetrosis alone.

Examination of the kidneys of these birds revealed virus distributions similar to those found in birds with P.R.C. 18 tumours.

From table 15, page 158 , it can be seen that birds injected with R.P.L. 12 contaminated with osteopetrosis, or with extracts from these tumours, developed a virus infection in the kidney, similar to that described in section IV of this thesis. Tissues of birds infected with R.S.V. contaminated tumours did not show a viral infection of the tissues. However, the most important result in table 15, page 158 , is the apparent viral infection of tissues in birds which had been infected with the R.P.L. 12 tumour strain contaminated with first osteopetrosis, and then Rous.

DISCUSSION

The aim of these experiments has been to show the ability of tumours to act as centres of virus multiplication and to explain the reported relationship of osteopetrosis and R.P.L. 12.

The description of the R.P.L. 12 tumour, given by Burmester (1946) does not agree with the appearance of the R.P.L. 12 tumour grown in this laboratory, even though it was obtained from Burmester's strain. The morphology and behaviour of the tumour is much more consistent with that expected of a neoplasm

of monocytic origin. It is possible that this tumour has, in fact, been incorrectly classified as a lymphoid tumour (Darcel and Negroni, 1954). If this is true, and R.P.L. 12 is of monocytic origin, then the phagocytic behaviour could account for the non-specific viral contamination. Olson's (1941) original isolation of the tumour from a bird with abnormally high monocytic and monoblastic content in the blood suggests a priori that the tumour could have been monocytic. However, the origin of the R.P.L. 12 tumour is of secondary importance, compared with its behaviour in the contamination experiments described.

Cell-free extracts of both P.R.C. 18, and R.P.L. 12, grown in birds showing clinical symptoms of osteopetrosis, both produce osteopetrosis after injection into day-old chicks. In both cases, the tumours used as sources of osteopetrosis virus could not be passaged by cell-free extracts. P.R.C. 18 appears to be carrying osteopetrosis as a passenger, and none of the experiments have shown an aetiological relationship between a viral agent and this tumour. R.P.L. 12 has also been shown to have the ability to act as a centre for osteopetrosis virus multiplication, both by electron microscope examination and passage experiments. Again, like P.R.C. 18, before and after contamination with osteopetrosis, R.P.L. 12 could only be passaged by cellular suspensions. Consequently, if uncontaminated R.P.L. 12 is ever grown in a bird which has an osteopetrosis virus infection, then subsequent passages of that tumour will be associated with osteopetrosis. If the osteopetrosis virus did

not replicate and grow in the tumour, passage of both osteopetrosis and R.P.L. 12 could not be maintained by concurrent transmission of uncontaminated tumour tissue, and infected blood: the rapid growth and fast passaging of the tumour would probably result in the virus being diluted out of the tumour tissue.

Since it has been shown in section IV of this thesis that osteopetrosis virus can multiply in birds without producing any clinical symptoms of the disease, then birds with this type of 'carrier' infection will usually be accepted as normal. Consequently, passage of R.P.L. 12 cells into osteopetrotic 'carrier' birds would produce tumours which were contaminated with virus. The resulting isolation of osteopetrosis virus from a tumour after a passage of this kind would be inexplicable, without a knowledge of the life cycle of the virus involved.

Contamination with R.S.V. indicated that this virus, too, can infect R.P.L. 12. An oncogenic virus of this type, which produces transformation, in the cells it infects, might be expected to show its presence by altering the infected tumour cell type. A careful examination of Rous contaminated R.P.L. 12 tumours was carried out, to try and detect any 'nests' of Rous Sarcoma type cells. No evidence for a change of cell type was found, which is rather unexpected, but corroborated by the apparent lack of R.S.V., demonstrated with the electron microscope. It must be concluded that R.S.V. is present in very small concentrations, and this is borne out

by the apparent diminution of the titre at each passage, indicated by the reduction in the positive reaction to cell-free extracts of contaminated R.P.L. 12, (tables 14, 15, pages 157, 158).

Double infection with both osteopetrosis and R.S.V. showed that more than one virus can contaminate R.P.L. 12 at the same time. In this case, the two viruses were demonstrated in cell-free extracts. R.S.V., extracted from these tumours, after several passages, was of a low titre, and often took a long time to establish itself and form a tumour. Consequently, osteopetrosis virus had time to become established in the kidney. Whilst the demonstration of osteopetrosis-like virus replicating in the kidney is not claimed to be a conclusive method of diagnosing osteopetrosis infection, many of the previous experiments have indicated that osteopetrosis virus does have an affinity for kidney cells. R.S.V. has not been found to replicate in the kidney in large numbers, like the osteopetrosis virus, and, similarly, birds infected with R.P.L. 12 contaminated with R.S.V., showed no kidney infection. Consequently, the presence of high concentrations of osteopetrosis-like particles in the kidney of birds with R.P.L. 12, grown in both osteopetrotic and Rous Sarcoma infected birds, probably indicates a double infection of the R.P.L. 12 tumour.

This double infection means that, if a tumour becomes infected with two viruses, both of which produce pathological conditions, then only the fast developing one may be apparent in cell-free extracts. This is an obvious and elementary point,

but it may help to explain some of the results of experiments, using R.P.L. 12 as experimental material, obtained in American laboratories. It seems likely that, had these experiments been continued, Rous virus might have been diluted out of the contaminated R.P.L. 12 tumour. If this had taken place, then osteopetrosis viruses in the doubly infected tumours would have been able to produce clinical symptoms. The implications of this type of change in the oncogenic potential of tumour extracts will be discussed in the General Discussion in the following pages, with particular reference to the leucosis group of viruses.

TABLE 12

ROUTINE PASSAGES OF P.R.C. 18

Number of birds inoculated with P.R.C. 18 cells	Number with solid P.R.C. 18 tumours	Number with osteopetrosis	Number examined by E.M.	Number with viruses in the kidney
71	48	8	8	8

Number of birds inoculated with cell-free extracts of P.R.C. 18 tumours	Number with solid P.R.C. 18 tumours	Number with osteopetrosis	Number examined by E.M.	Number with viruses in the kidney
10	0	3	10	7

TABLE 13

EXPERIMENTS WITH THE R.P.L. 12 TUMOUR,

AFTER PASSAGE IN BIRDS INFECTED WITH OSTEOPETROSIS

Osteopetrotic birds inoculated with R.P.L. 12 cells	Birds with R.P.L. 12 tumours
2	2

One of the R.P.L. 12 tumours was used for further passage in R.S.V. infected birds (page 158.), and the other was passaged as follows:

Passage	Birds inoculated with R.P.L. 12 cells	Birds with R.P.L. 12 tumours	Birds with viruses in the kidney	Birds inoculated with cell-free extracts of R.P.L. 12	Birds with R.P.L. 12 tumours	Birds with viruses in the kidney
I	6	6	6	6	0	4
II	6	6	5	6	0	4
III	6	6	5	5	0	3

TABLE 14

EXPERIMENTS WITH THE R.P.L. 12 TUMOUR, AFTER PASSAGE IN BIRDS PREVIOUSLY INOCULATED WITH ROUS SARCOMA CELLS

Number of birds inoculated with Rous Sarcoma cells followed by R.P.L. 12 cells	Number of birds with Rous Sarcoma	Number of birds with R.P.L. 12 tumours
4	4	4

One of the R.P.L. 12 tumours was passaged as follows:

Passage	Birds inoculated with R.P.L. 12 cells	Birds with R.P.L. 12 tumours	Birds with viruses in the kidney	Birds inoculated with cell-free extracts of R.P.L. 12	Birds with R.P.L. 12 tumours	Birds with Rous tumours	Birds wit viruses i the kidne
I	6	6	0	5	0	5	0
II	7	7	0	5	0	5	0
III	6	6	0	6	0	4	0

TABLE 15

EXPERIMENTS WITH THE R.P.L. 12 TUMOUR AFTER PASSAGE IN OSTEOPETROTIC BIRDS FOLLOWED BY PASSAGE IN BIRDS WITH ROUS SARCOMATA

An R.P.L. 12 tumour passaged in osteopetrotic birds (see table 13), was used initially as a source of cells.

Birds inoculated with Rous cells followed by R.P.L. 12 cells	Birds with Rous tumours	Birds with R.P.L. 12 tumours
4	4	4

One of the R.P.L. 12 tumours was passaged as follows:

Passage	Birds inoculated with R.P.L. 12 cells	Birds with R.P.L. 12 tumours	Birds with viruses in the kidney	Birds inoculated with cell-free extracts of R.P.L. 12	Birds with R.P.L. 12 tumours	Birds with Rous tumours	Birds with viruses in the kidney
I	6	6	6	6	0	2	3
II	6	6	4	5	0	2	2
III	6	6	5	6	0	1	3

PLATE 36

P.R.C. 18 cells showing intercellular particles.

PLATE 36

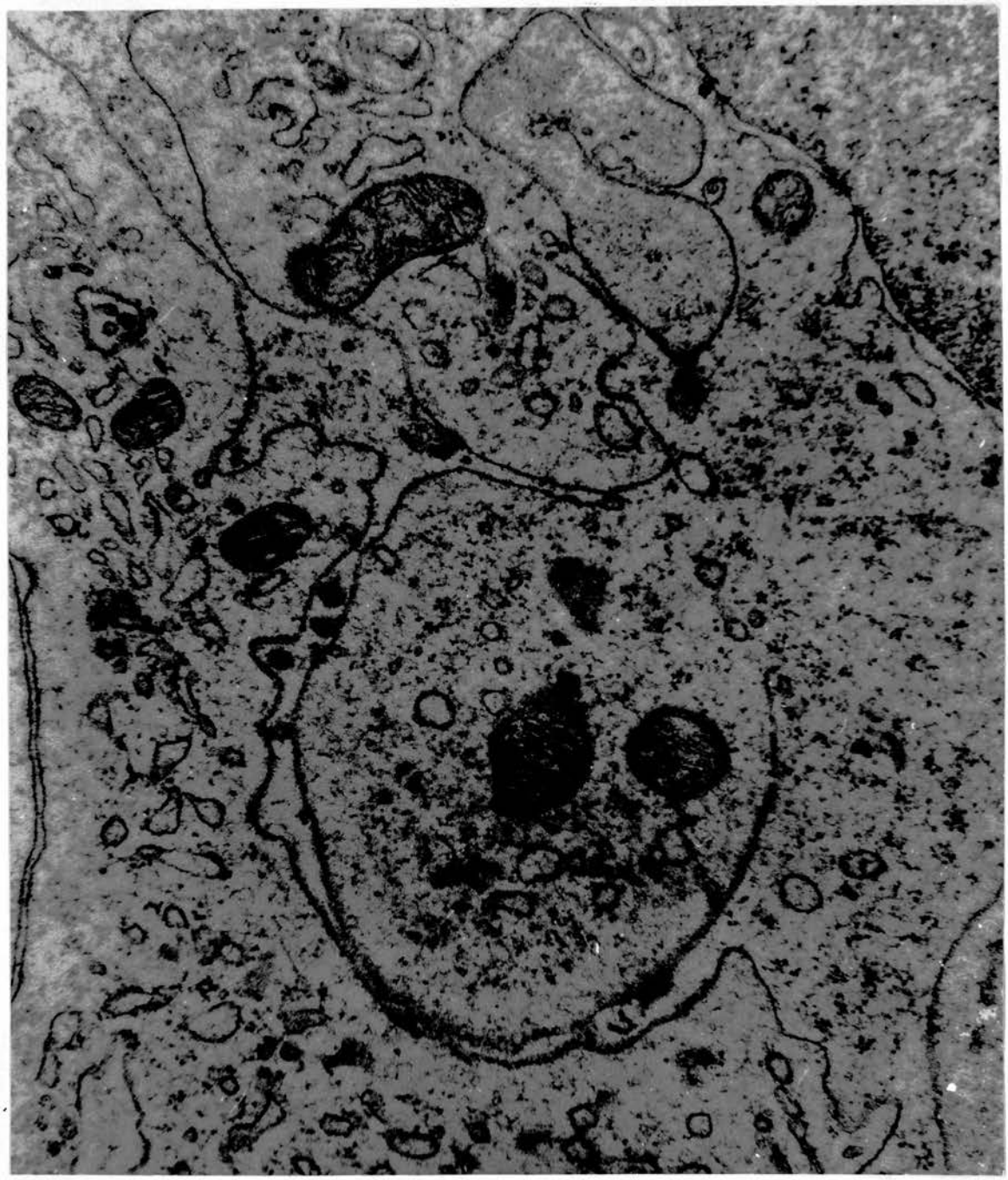


PLATE 37

High power micrograph of part of the previous plate. The typical osteopetrosis-type of particle can be seen.

PLATE 37

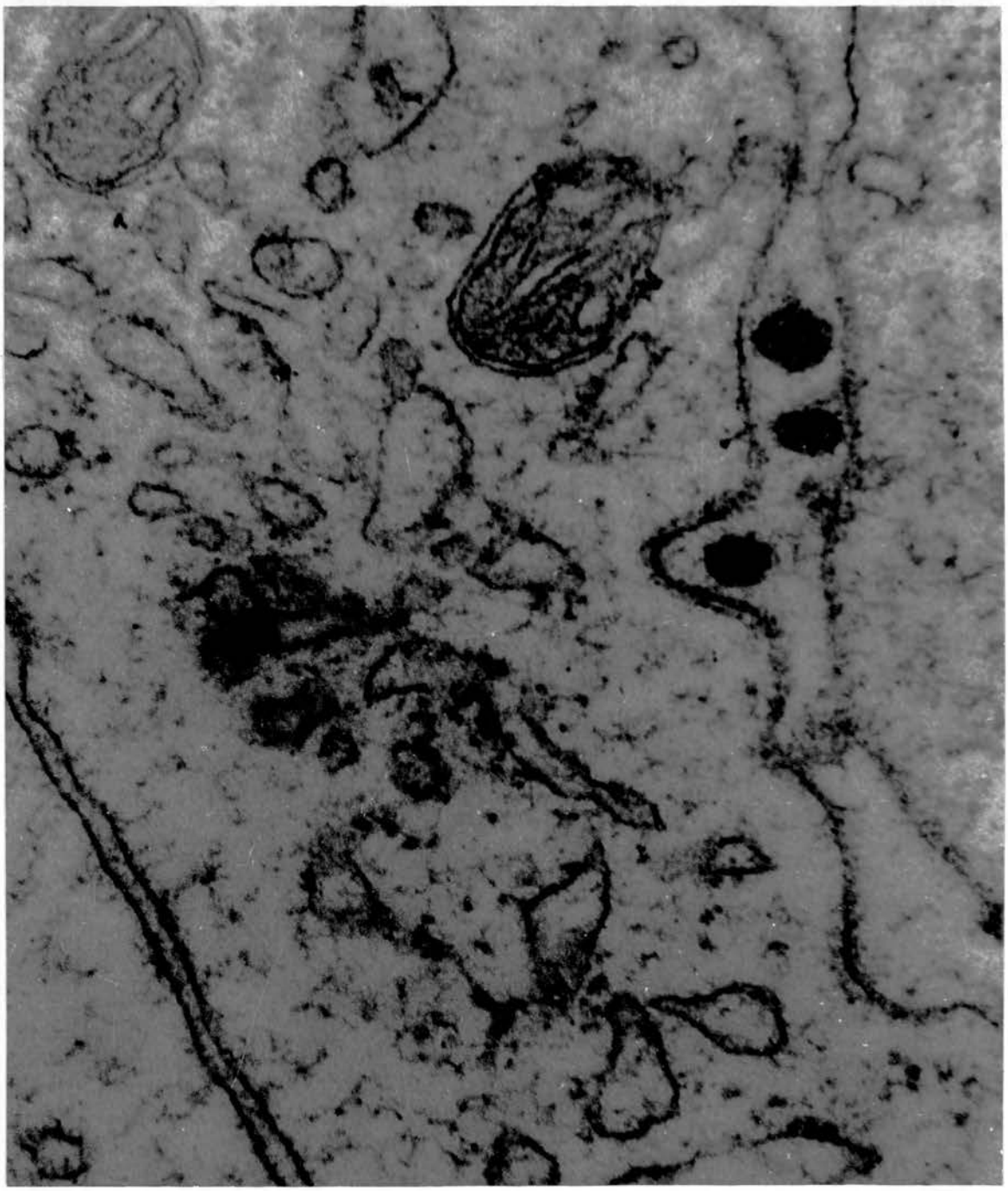


PLATE 38

Cells of an R.P.L. 12 tumour.

PLATE 38

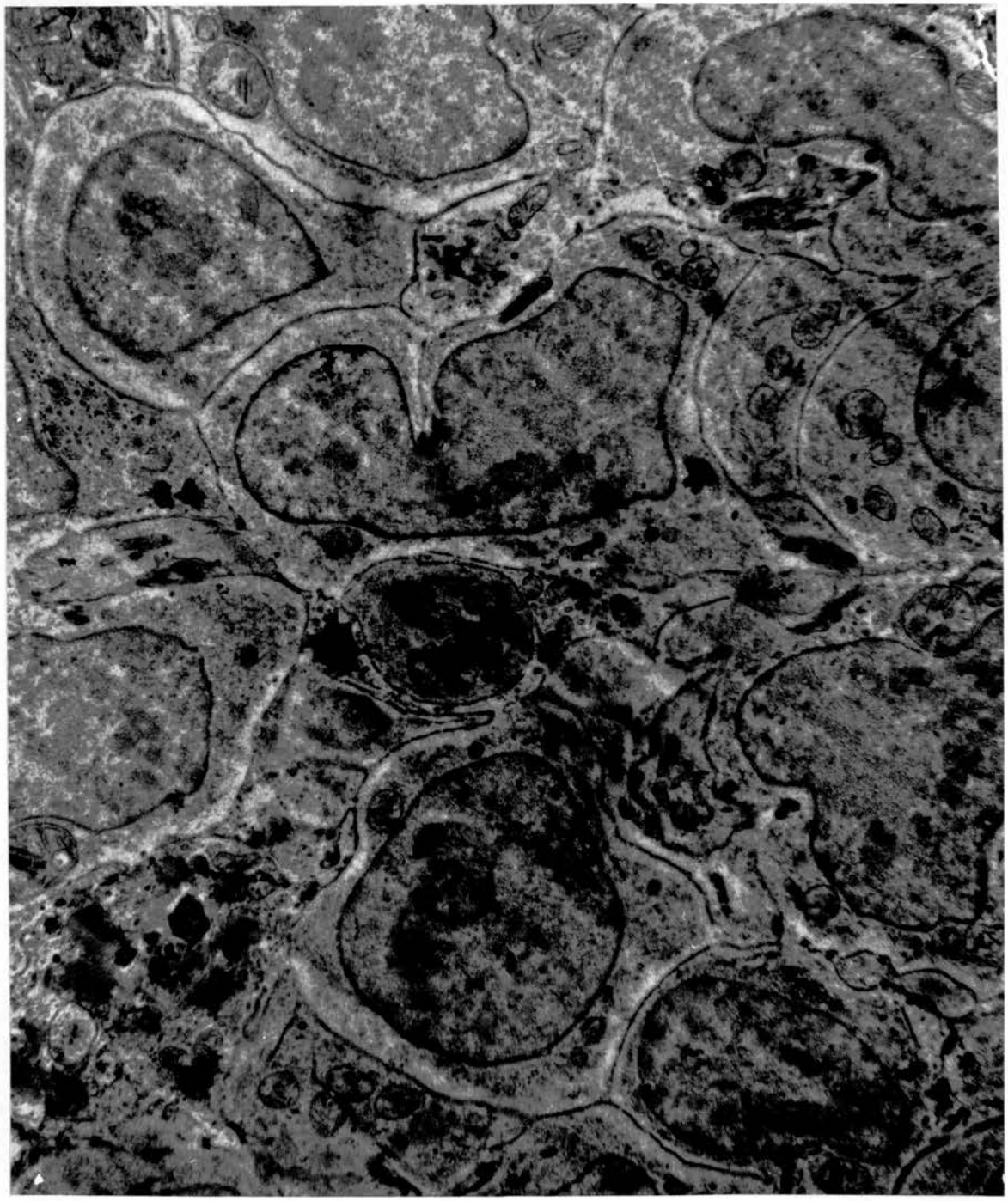


PLATE 39

The cytopathogenic viruses found in the R.P.L. 12 tumour; a group of these viruses can be seen in the upper left part of this plate.

PLATE 39

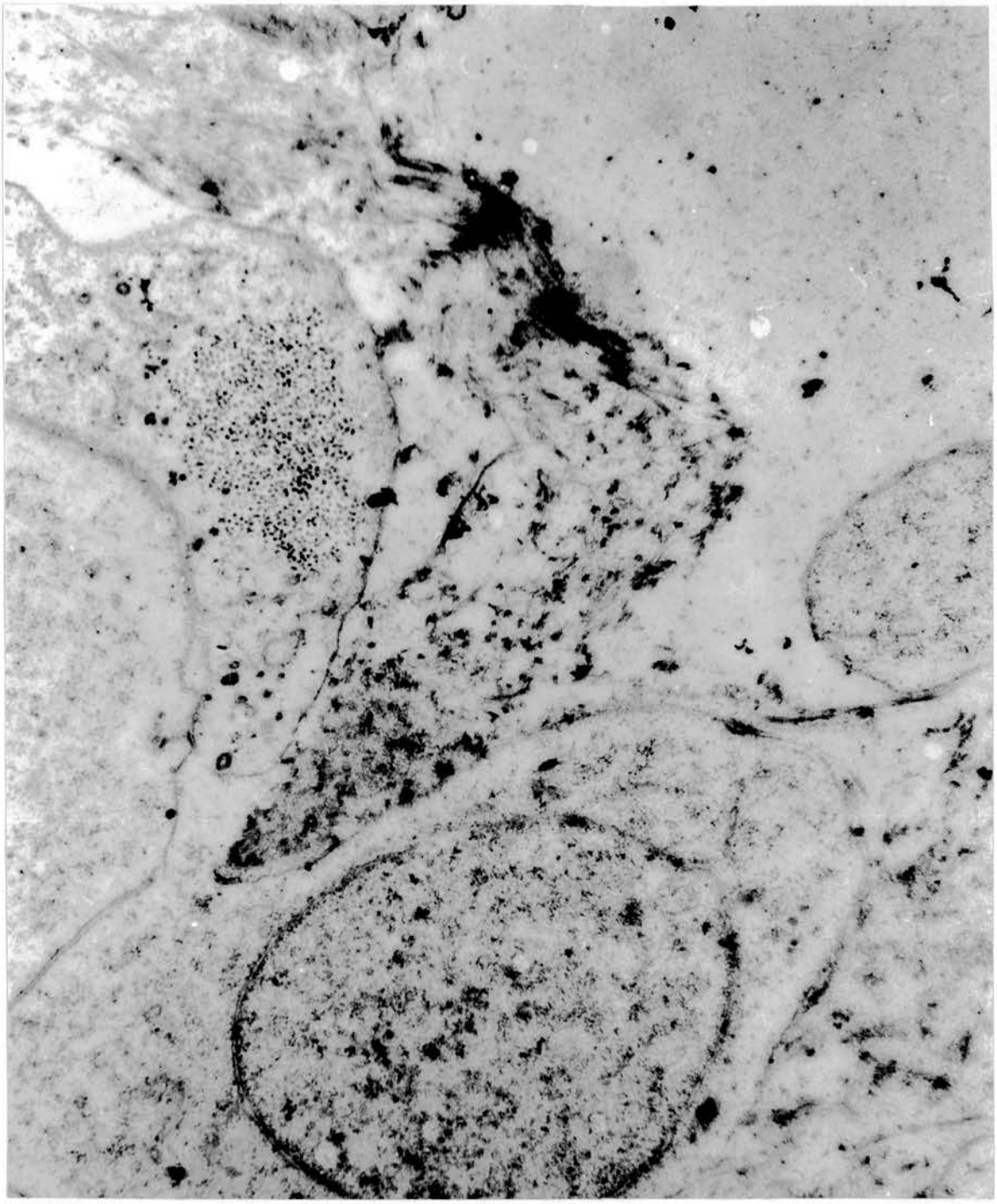


PLATE 40

The cytopathogenic viruses found in R.P.L. 12 associated with a cell which is obviously degenerating or perhaps dead. Nuclear structure appears to be degenerating and the cytoplasm contains many viruses.

PLATE 40

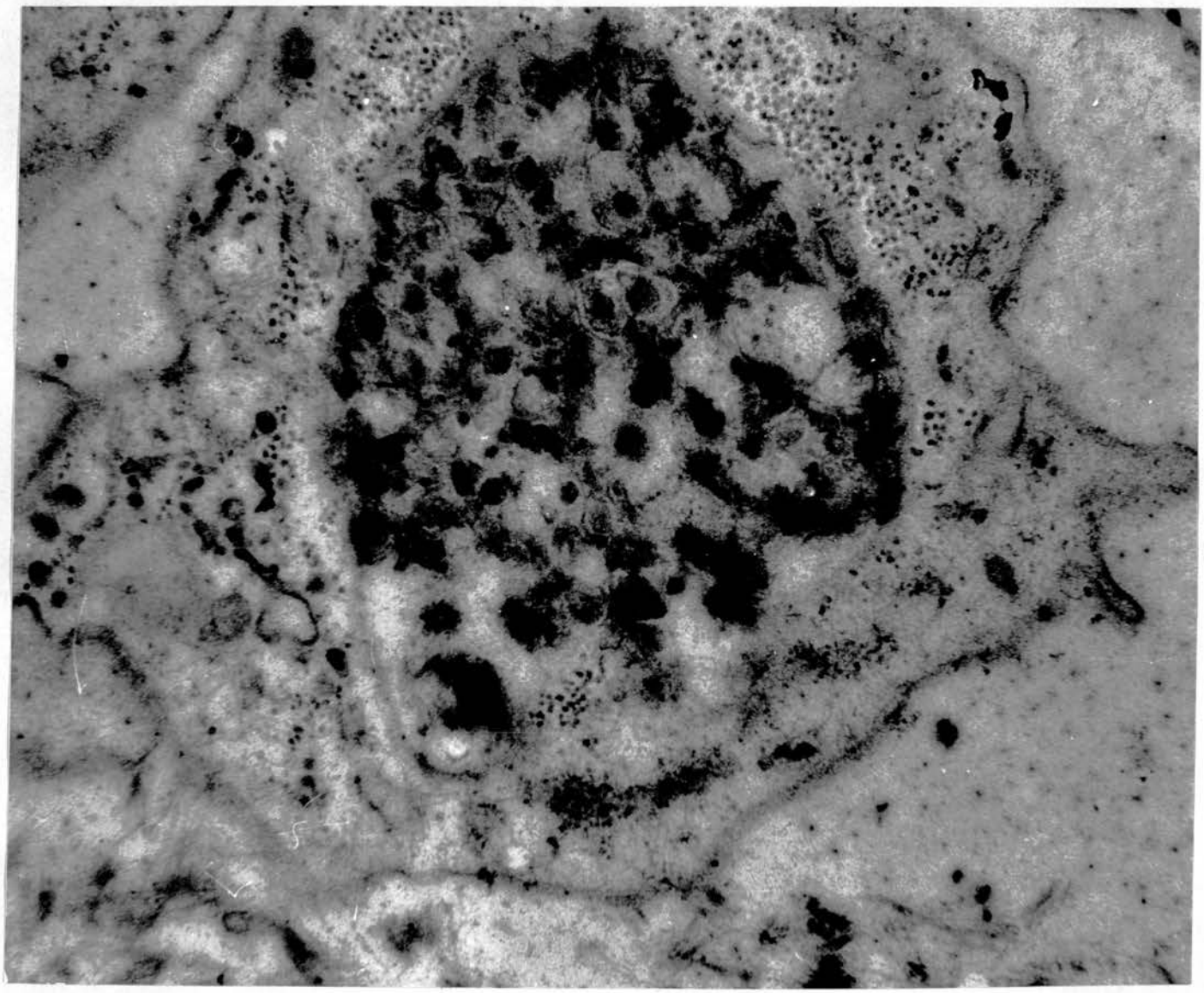
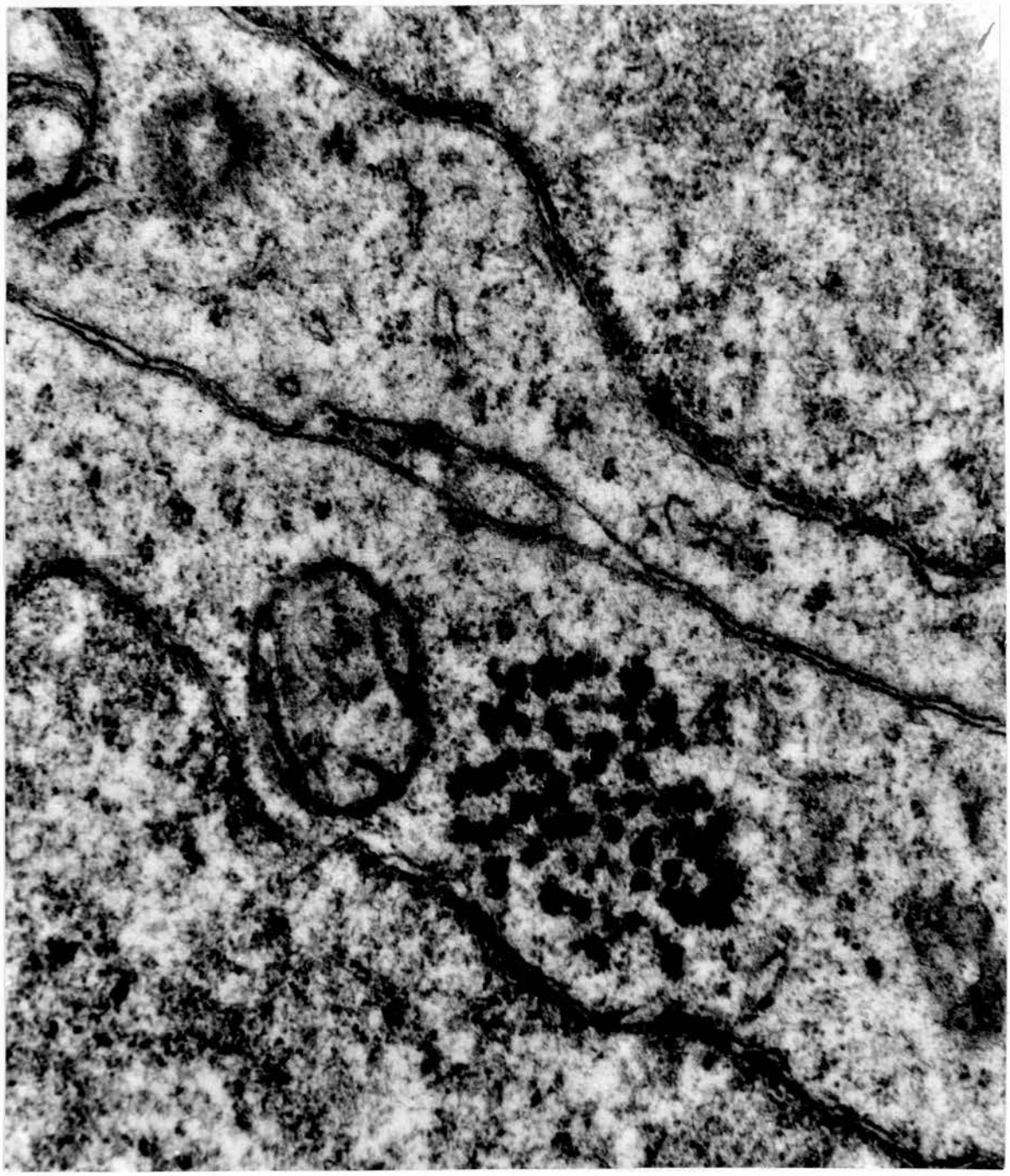


PLATE 41

A group of cytoplasmic particles of the type found in R.P.L. 12 and P.R.C. 18 tumour cells.

PLATE 41



SECTION VII

GENERAL DISCUSSION

The transmissibility of osteopetrosis has been established by several authors (Jungherr and Landauer, 1938; Burmester, 1946; Holmes, 1958; Campbell et al, 1964), but the characteristics of the transmissible particle have not previously been described. In the present study, preliminary filtration experiments established that millipore filters, with a pore size of less than 1,000Å, retain the infective agent. This agrees with Holmes' (1958) observation that normal bacterial filters failed to retain the agent, and that the probable particle size suggested a virus. If the particle responsible for osteopetrosis was not less than 1,000Å, and not more than 2,200Å, as indicated by the millipore filters, the inference is that the particle is within the size range of a leucosis virus, (Beard et al, 1950). This immediately suggests the view expressed by Burmester (1946) that osteopetrosis may be a feature of the response to leucosis virus infections, may in some way be due to the similarity in size of the particles. The subsequent survey of periosteum and muscle lesions of osteopetrosis infected birds showed the presence of many small spheroidal particles of approximately 1,100Å in diameter. This agreed with the filtration experiments, and it was assumed that these particles were responsible for osteopetrosis. A detailed examination of the particles in the periosteum, the muscle lesions and the kidney, which were the tissues in which the highest concentration of virus particles was found, showed that they were all

morphologically similar. Budding from the cell membrane of infected cells has been described, and appears to be a general characteristic of replication of the virus in all the different tissues examined. Only very occasionally were intracellular particles seen, in the macrophages of the blood, and in the Kupffer cells of the liver, and this was probably due to the phagocytic character of the cells, rather than to the viruses' ability to develop intracellularly. The extensive histochemical examination confirmed that the particles observed were viruses with nucleoids, about 800Å in diameter, composed of R.N.A., with two surrounding membranes of lipoprotein. Further evidence of the lipoprotein nature of this coat, and confirmation of the role of a particle of this type in the aetiology of osteopetrosis, was shown by the reduction in infectivity of the virus preparations after treatment with organic solvent, (Drayton, 1960a,b).

This virus 'type' has been associated with cells of many different avian virus-induced conditions: e.g., the Murray-Begg tumour (Haguenau et al, 1955; Roulier et al, 1956), Fujinami tumour (Mannweiler and Bernhard, 1958), and Rous tumours (Gaylord, 1955; Haguenau, 1960a; Haguenau and Beard, 1962); in myeloblastosis leukaemia (B.A.I. Strain A) (Dmochowski et al, 1958a,b; Parsons et al, 1959; Bonar et al, 1959), and erythroblastosis (leukaemia R) (Dmochowski et al, 1958a; Heine et al, 1961); in association with the disease caused by the transmissible R.P.L. 12 lymphoid tumour (Dmochowski and Grey, 1958; Dmochowski et al, 1959a,b), in

the cells of the nephroblastoma (Dmochowski et al, 196; Heine et al, 1963) caused by the B.A.I. Strain A and also those of the thymus, pancreas and other tissues of birds with myeloblastosis (Heine et al, 1963; de Thé et al, 1962). They have also been observed in purified preparations of B.A.I. Strain A (Bernhard et al, 1958) and Rous Sarcoma virus (Epstein, 1956, 1960; Epstein and Holt, 1958) and R strain (Bernhard et al, 1958).

The morphology of the particles isolated from, or observed in, all these different conditions was similar, and the slight differences in size between the various particles may have been due to variations in electron microscopical preparative techniques. Beard (1963) considers this variation in shape and size to be due to the loosely bounded nucleoid. This results in different estimations in the size of B.A.I. Strain A virus being calculated from sections viewed in the electron microscope (Bernhard, 1958), in shadowed preparations (Sharp et al, 1952) and from sedimantation data (Sharp and Beard, 1954). However, Beard (1963) concludes that the exact measurement of this type of particle is of little significance, since the particles must change their shape, depending on their degree of hydration. Osteopetrosis virus particles can be regarded as being very similar to the particles listed above, which have been associated with avian virus-induced tumours.

Unlike the <u>in vivo</u> assay methods used for R.S.V., (Carr and Harris, 1951), where a minimal infective dose can be estimated from the effect of serial dilutions on day-old chicks,

the assay of the leucosis viruses has been handicapped by the relatively large incubation period required for one or more of the neoplasms to develop, and by the variation in response. As a result, bio-assays (Piraino et al, 1963), have not proved successful. At the time this work was begun, the only relatively reproducible method of assaying leucosis viruses, with the exception of myeloid leucosis (Mommaerts et al, 1954), was by techniques based on the interference phenomena, first described by Rubin (1960), and subsequently developed by Vogt and Rubin (1963), which have been described in section IV. Attempts were made to develop an interference assay technique for osteopetrosis virus, since in vivo assays of this virus, resulting from infection of day-old chicks, took at least three months. Unfortunately, the lack of tissue culture facilities prevented the development of an infective centre, or interference assay. However, no detectible transformation was observed seven days after the infection of chick fibroblast cultures with partially purified osteopetrosis virus preparations. The C.A.M. response to osteopetrosis virus preparations was inconsistent, and no explanation for this variability in response could be elucidated. However, virus preparations injected onto the C.A.M. occasionally resulted in the multiplication of osteopetrosis virus to such an extent that, after three days, the osteopetrosis infected C.A.M. was immune to subsequent R.S.V. infection. Although the response was variable, it can be concluded that osteopetrosis virus can be included in the R.I.F. group of viruses.

Recently, Sarma et al (1964) have developed an assay method for the detection of the avian leucosis viruses, by using complement fixation techniques. This method is based on the ability of Schmidt-Ruppin Rous Sarcoma virus (Ahlstrom and Forsby, 1962) to produce subcutaneous fibro-sarcomata, in hamsters and Guinea-pigs, from which infective virus particles cannot be isolated. These non-virus producing tumours, however, induce complement-fixing antibodies, in hamsters and Guinea-pigs, which are reactive, not only with the homologous virus, but with other leucosis-type viruses, such as myeloid leucosis, erythroleucosis, R.P.L. 12, R.I.F., and the Bryan strain of Rous Sarcoma virus (Huebner et al, 1963). Consequently, a source of serum, uncontaminated with non-virus specific chicken antibodies, is readily available. End point assays were obtained by inoculating serial dilutions of virus into chicken embryo fibroblast cultures, grown in petri dishes. The development of complement fixation viral antigens was then assayed, after one or two cell transfers, by a micro-complement fixation test, (Sever, 1962; Huebner et al, 1963).

This type of assay has advantages over previous R.I.F. assay techniques, in that it is quicker and easier to carry out, and virus-specific antiserum can be obtained.

This technique was not attempted due to lack of culturing facilities and time, but it is probable that the osteopetrosis virus would also share the common antigens found in the leucosis-type of viruses already mentioned.

On the basis that no leucotic condition developed in either control or experimental birds, the assumption has been made that the osteopetrosis viruses studied formed a homologous population. It would have been a great advantage if this point could have been confirmed by cloning the virus and passaging a cloned population (Hanafusa et al, 1963). Unfortunately, these facilities for cloning osteopetrosis virus were not available.

However, the life-cycle of the osteopetrosis virus, determined by electron microscopy, reveals a consistent pattern, suggesting the uniformity of the inoculated virus preparations. The study of the life cycle reveals that the normal course of the infection is unlikely to result in leukaemia, since the primary site of virus multiplication appears to be the kidney. This suggestion is based on the observation that the first detectable virus, three weeks after injection of osteopetrosis virus into day-old chickens, is in this organ. Other tissues examined do not show demonstrable virus until times ranging from four weeks for the spleen, to six months or a year for the muscle lesions, after the initial inoculations. The replication of virus in large quantities in the kidney is not altogether unexpected, as several authors have reported the involvement of the kidney in avian tumour conditions associated with viruses (Harris, 1963). Foulds first reported the production of kidney carcinomata in fowls infected with reticulendothelioma, and this was confirmed by Carr (1959), and Thorell (1958). Carr (1959) concluded that Rous Sarcoma

virus I, Duran-Reynals-D, Sarcomata 2, 3 and 4 of the Poultry Research Centre, and the non-virus induced sarcomata G.R.C.H. 16, and Carr and Campbell's myeloid tumour, did not produce these kidney carcinomata. MH_2 endothelioma is reported as producing renal carcinomata like those produced by E.S.4 (Carr, 1960), and Chouroulinkov and Riviere (1959) suggest that these are primary tumours and not metastitic. Dmochowski et al (1961) and Burmester et al (1959a) have also claimed that myeloblastosis can produce primary or transplantable tumours in the kidney, which were shown to contain both intracellular and intercellular virus particles. These particles were reported to have an overall diameter of about 1,000Å and nucleoids of between 625 and 850Å in diameter, which is consistent with the size of the leucosis type of virus particle. However, none of the descriptions of kidney involvement have included the extent of virus growth in this organ. It is possible that many of the chicken viruses replicate in the kidney, without normally producing gross lesions, in the same way as osteopetrosis virus. This might explain the frequent findings of tumours in this organ, and the infectivity of the urine and faeces in many of the leucotic conditions, (Foulds, 1934).

The extensive distribution of the osteopetrosis virus throughout the tissues of infected birds examined (including kidney, liver, spleen, marrow, periosteum, muscle lesions, pancreas, blood and bone marrow), suggests that this virus is also responsible for the high incidence of lesions, other than bone lesions, associated with osteopetrosis. It has been

shown in these studies that the osteopetrosis virus normally replicates in the kidney cells, and only occasionally produces bone hyperplasia and tumours in the muscles and kidney. In one experiment, 74.4% of all birds examined were shown to have osteopetrosis virus growing in the kidney, but without any accompanying pathological bone conditions. A situation of this type would explain the virus-like particles found in normal chicken tissue, (Benedetti, 1957; Karrer, 1960; Rubin, 1960; Zeigel, 1961), and the suggestion by Rubin (1960) that R.I.F. is widely distributed by viraemic 'carriers', which are apparently normal birds. Although it is not suggested, from the evidence presented here, that osteopetrosis may be responsible for the distribution of R.I.F., it may be inferred that the leucoses conditions, like osteopetrosis, extensively infect the host birds' tissues, have an affinity for kidney tissues, and can produce viraemia without the clinical signs of the disease (Burmester, 1962). It is not surprising that these similarities in the behaviour of the leucosis and osteopetrosis viruses resulted in a confusion of the aetiological relationships.

The examination of the P.R.C. 18 tumour has indicated the passenger virus relationship of osteopetrosis viruses with this tumour. A similar explanation was presented in order to explain the presence of osteopetrosis virus in R.P.L. 12 tumour tissue, grown in osteopetrotic birds.

The origin and classification of the R.P.L. 12 tumour has not been considered in detail, and the suggestion that this tumour is of monocytic origin, rather than lymphocytic, is

made tentatively. However, this does agree with Darcel and Negroni's (1954) similar suggestion, and fits Olson's (1941) data. The R.P.L. 12 tumour grown in osteopetrotic birds was carefully examined to determine the association of the osteopetrosis virus with the tumour cells. It was surprising that the examination of the R.P.L. 12 tumour did not reveal any virus particles before contamination was attempted, since Dmochowski et al (1959b) has reported the presence of intracellular particles 720Å in diameter, in birds with the disease induced by R.P.L. 12. Similarly, no transmissible conditions could be induced with cell-free extracts from the R.P.L. 12 tumour. Although this is contrary to Burmester's many reports of the infectivity of R.P.L. 12 extracts, it is consistent with the observations of several workers in this country (Campbell, 1961; Darcel and negroni, 1954; Carr, 1961).

The R.P.L. 12 tumour, passaged at the Poultry Research Centre, has been considered to be of the same cell type as the tumour maintained by Burmester et al (1946). After passage in osteopetrotic birds, it was subsequently shown to be contaminated with osteopetrosis virus. Burmester et al (1946) do not record being able to detect an osteopetrogenic agent associated with R.P.L. 12, until fifty to two hundred passages of the original tumour material have been carried out. Since osteopetrosis has been reported (Burmester and Denington, 1947) in 'a few birds' of the flock used to passage R.P.L. 12, it would seem that carriers of osteopetrosis virus almost certainly occur in Burmester's flock. The persistence of osteopetrosis

virus in the R.P.L. 12 tumour reported by Burmester agrees with the findings reported in this work, and the behaviour of passenger viruses in general, as described in the introduction. A similar argument can be used to explain the association of osteopetrosis virus with R.P.L. 18 and R.P.L. 21 (Burmester, 1947a). After nineteen years of passaging R.P.L. 12, Burmester et al (1960) isolated erythroblastosis from it, as well as lymphoid leucosis and osteopetrosis. It has been reported in these present studies that the R.P.L. 12 tumour can be experimentally contaminated with two separate viruses, which would bring about an anaolgous situation to that reported by Burmester. It is assumed that the bird which grew the R.P.L. 12 tumour, from which the extract inducing erythroblastosis was obtained, did not show any clinical signs of erythroblastosis. However, if this bird had been a 'carrier' of erythroblastosis virus, it is probable that the R.P.L. 12 tumour could have become secondarily infected with that virus.

Gross et al (1959), and Burmester et al (1959b), have shown that extracts of R.P.L. 12 tumours induce four different neoplastic conditions. This has been explained by Burmester et al (1960a) as being due to the variation in response of the chickens used, rather than to the inoculation of a heterogenous preparation of several different viruses. They explained the varying response by suggesting that the routes of inoculation, the age of the birds, and the size of the dose, were the critical factors determining the response. It is well known that the

susceptibility of different birds to the same dose of a virus infection can vary, and this may be related to the reported variation in virus content of extracts of routinely passaged R.P.L. 12 tumours. However, although the age of the recipient birds is important for the development of osteopetrosis, the dose has not been shown to affect the type of neoplastic conditions induced. On this evidence, the concept of the multipotency of a single virus entity being responsible for osteopetrosis, erythroleucosis, lymphoid leucosis, and myeloid leucosis, is unlikely. A more likely explanation is that a group of viruses, which have very similar morphological, physical, chemical and antigenic characteristics, are individually responsible for each disease. The confusion is probably due to the natural background incidence of all the reported conditions in many of the experimental flocks, due to carrier birds, and to the difficulties in the separation of viruses into 'cloned' strains.

B I B L I O G R A P H Y

ABOLINS, L., (1952): Exp. Cell Res., 3; p. 1.

AGEENKO, A. I., (1960): Prob. Oncol. (U.S.S.R.) (Eng. trans.), 6; p. 409-423.

AHLSTROM, C. G., FORSBY, N., (1962): J. Exp. Med., 115; p. 839-852.

ALMEIDA, J. D., HASSELBACK, R. C., HAM, A. W., (1963): Science, 142, No. 3598; p. 1487-1489.

ANDERSON, S. G., (1959): in "The Viruses" (Biochemical, Biological and Biophysical Properties), vol. 3; p. 21: ed., F. M. Burnet, W. M. Stanley. Academic Press, Inc., New York and London.

ANDREWS, C. H., (1964): Brit. Med. J., 5384; p. 653-658.

ANDREWS, C. H., HORSTMAN, D. M., (1949): J. Gen. Microbiol., 3; p. 290.

ARMSTRONG, G. R., (1956): Exp. Cell Res., 11; p. 640.

BALUDA, M. A., (1962): in "Symposia on Quantitative Biology", vol. 27; p. 415-426. Biological Laboratory, Cold Spring Harbor, New York.

BALUDA, M. A., GOETZ, I. E., (1961): Virology, 15; p. 185-199.

BATHER, R., (1954): Ph.D. Thesis, Edinburgh University.

BAUER, H., (1933): Z. Mikroskop. Anat. Forsch., 33; p. 143.

BEARD, J. W., (1963): in "Advances in Cancer Research", vol. 7: ed., A. Haddow, S. Weinhouse. Academic Press, Inc., New York and London.

BEARD, D., ECKERT, E. A., CZAKY, T. Z., SHARP, D. G., BEARD, J. W., (1950): Proc. Soc. Exp. Biol. Med., 75; p. 533-536.

BENSLEY, C. M., (1939): Stain Tech., 14; p. 47.

BELL, D. J., CAMPBELL, J. G., (1961): J. Comp. Path., 71; p. 85.

BENEDETTI, E. L., (1957): Bull. Cancer, 44; p. 473-482.

BURMESTER, B. R., GENTRY, R. F., (1954): Cancer Res., 14; p. 34-42.

BURMESTER, B. R., WALTER, W. G., GROSS, M. A., FONTES, A. K., (1959a): J. Nat. Cancer Inst., 22; p. 103-127.

BURMESTER, B. R., WALTER, W. G., GROSS, M. A., FONTES, A. K., (1959b): J. Nat. Cancer Inst., 23(2); p. 277-291.

BURMESTER, B. R., FONTES, A. K., WALTER, W. G., (1960a): J. Nat. Cancer Inst., 24; p. 1423.

BURMESTER, B. R., SHARPLESS, G. R., FONTES, A. K., (1960b): J. Nat. Cancer, Inst., 24; p. 1443-1447.

BURMESTER, B. R., WALTER, W. G., (1961): J. Nat. Cancer Inst., 26(2); p. 511.

BURNET, F. M., LUSH, D., (1940): Aust. J. Exp. Biol. Med. Sci., 18; p. 141.

CAMPBELL, J. G., (1961): Brit. Vet. J., 117; p. 316-325.

CAMPBELL, J. G., (1963a): Proc. Roy. Soc. Edin. (B), 67; p. 303.

CAMPBELL, J. G., (1963b): Personal Communication.

CAMPBELL, J. G., YOUNG, D. E., CARR, J. G., (1964): J. Comp. Path., 74; p. 263.

CARR, J. G., (1959): Virology, 8; p. 269-270.

CARR, J. G., (1960): Brit. J. Cancer, 14; p. 77.

CARR, J. G., (1961): Personal Communication.

CARR, J. G., HARRIS, R. J. C., (1951): Brit. J. Cancer, 5; p. 83-94.

CARR, J. G., CAMPBELL, J. G., (1958): Brit. J. Cancer, 12; p. 632-635.

CAULFIELD, J. B., (1957): J. Biophys. Biochem. Cytol., 3; p. 827.

CHOUROULINKOV, I., RIVIERE, M. R., (1959): Bull. Assoc. Franc. Cancer, 46; p. 722.

COSSLETT, V. E., (1951): in "Practical Electron Microscopy". Butterworth's, London.

CULLING, C. F. A., (1963): in "Handbook of Histopathological Techniques". Butterworth's, London.

DALTON, A. J., HAGUENAU, F., MOLONEY, J., (1964): Virology,

DANIELLI, J. F., (1953): in "Cytochemistry: A Critical Approach". Wiley, New York.

DARCEL, C. Le Q., NEGRONI, G., (1954): in "Leukaemia Research", p. 61-69: ed., G. E. W. Wolstenholme, M. P. Cameron, J. Etherington. Ciba Foundation Symposium.

DHALIWAL, S. S., (1959): Brit. J. Cancer, 13; p. 685-693.

DI STEPHANO, H. S., (1952): Science, 115; p. 316.

DMOCHOWSKI, L., (1963): Progr. Exp. Tumour Res., 3; p. 35.

DMOCHOWSKI, L., GREY, C. E., (1958): Blood, 13; p. 1017-1042.

DMOCHOWSKI, L., GREY, C. E., BURMESTER, B. R., FONTES, A. K., (1958a): Proc. Soc. Exp. Biol. Med., 98; p. 662-665.

DMOCHOWSKI, L., GREY, C. E., BURMESTER, B. R., WALTER, W. G., (1958b): Proc. Soc. Exp. Biol. Med., 98; p. 666-669.

DMOCHOWSKI, L., GREY, C. E., BURMESTER, B. R., GROSS, M. A., (1959a): Proc. Soc. Exp. Biol. Med., 100: p. 514-516.

DMOCHOWSKI, L., GREY, C. E., BURMESTER, B. R., (1959b): Proc. Soc. Exp. Biol. Med., 100; p. 517-519.

DMOCHOWSKI, L., GREY, C. E., BURMESTER, B. R., WALTER, W. G., (1961): Texas Repts. Biol. Med., 19; p. 545-579.

DRAYTON, H., (1960a): Ph.D. Thesis, Edinburgh University.

DRAYTON, H., (1960b): Brit. J. Cancer, 14; p. 306.

DURAND, A., (1961): Personal communication.

ENGELBRETH-HOLM, J., ROTHE-MEYER, A., (1932): Acta. Path. Microbiol. Scand., 9; p. 293-312.

EPSTEIN, M. A., (1956): Brit. J. Cancer, 10; p. 33-48.

EPSTEIN, M. A., (1960): J. Nat. Cancer Inst. Symp., No. 4; p. 53.

EPSTEIN, M. A., (1964): Paper read at the meeting of "European Tumour Virologists", Royaumont, Paris.

EPSTEIN, M. A., HOLT, S. J., (1958): Brit. J. Cancer, 12; p. 363-369.

FARQUHAR, M. G., PALADE, G. E., (1959): Anat. Rec., 133; p. 378.

FARQUHAR, M. G., PALADE, G. E., (1960): J. Biophys. Biochem. Cytol., 7; p. 297-304.

FARQUHAR, M. G., PALADE, G. E., (1961): J. Exp. Med., 114; p. 699-716.

FARQUHAR, M. G., PALADE, G. E., (1962): J. Cell Biol., 13; p. 55-87.

FONTES, A. K., BURMESTER, B. R., WALTER, W. G., ISELER, P. E., (1958): Proc. Soc. Exp. Biol. Med., 97; p. 854-857.

FOULDS, L., (1934): Eleventh Scientific Report of the Imperial Cancer Research Fund; p. 1.

FREEMAN, J. A., SPURLOCK, B. O., (1962): J. Cell Biol., 13; p. 437-443.

FRIEND, C., (1957): J. Exp. Med., 105; p. 307-318.

FRIEND, C., HADDAD, J. R., (1960): J. Nat. Cancer Inst., 25; p. 1279-1289.

GAYLORD, W. H., Jr., (1955): Cancer Res., 15; p. 80-83.

GIBBONS, I. R., GRIMSTONE, A. V., (1960): J. Biophys. Biochem. Cytol., 7; p. 697.

GIMMY, J., GRAFFI, A., (1961): Arch. Geschwulstforsch, 18; p. 37-49.

GLAUERT, A. M., GLAUERT, R. H., (1958): J. Biophys. Biochem. Cytol., 4; p. 191.

GLICK, D., (1949): in "Techniques of Histo-cyto-chemistry". Inter. Sciences, New York and London.

GOMORI, G., (1952): in "Microscopic Histochemistry", University of Chicago Press.

GOTTSCHALK, R. G., (1946): Cancer Res., 6; p. 270-277.

GRAFFI, A., (1957): Ann. N.Y. Acad. Sci., 68; p. 540-558.

GREENWOOD, A. W., CARR, J. G., (1951): Paper read at "World's Poultry Science Association Conference, Paris".

GROSS, L., (1950): Cancer, 3; p. 1073-1087.

GROSS, L., (1957): Acta. Haematol., 15; p. 273.

GROSS, M. A., BURMESTER, B. R., WALTER, W. G., (1959): J. Nat. Cancer Inst., 22; p. 83-101.

HAGUENAU, F., (1960a): Compt. Rend. Acad. Sci., 250; p. 1477-1479.

HAGUENAU, F., (1960b): J. Nat. Cancer Inst., 24; p.873-880.

HAGUENAU, F., (1960c): Nat. Cancer Inst. Mono., 4; p. 211-249.

HAGUENAU, F., ROUILLER, C., LACOUR, F., (1955): Bull. Cancer, 42; p. 350-357.

HAGUENAU, F., BEARD, J. W., (1962): in "Ultrastructures in Biological Systems Sr.", vol. I, p. 1-59: ed., A. J. Dalton, F. Haguenau. Academic Press, New York and London.

HALE, A. J., (1957): Int. Rev. Cytol., 6; p. 193.

HALL, B. V., (1957): Am. Heart J., 54; p. 1-9.

HALL, W. J., BEAN, C. W., POLLARD, M., (1941): Am. J. Vet. Res., 2; p. 272-279.

HANAFUSA, H., HANAFUSA, T., RUBIN, H., (1963): Proc. Nat. Acad. Sci. U.S., 49; p. 572-58o.

HARRIS, R. J. C., (1960): in "Cancer Progress": ed., R. W. Raven. Butterworth's, London.

HARRIS, R. J. C., (1963): in "Cancer Progress": ed., R. W. Raven. Butterworth's, London.

HEINE, U., BEAUDREAU, G. S., BECHER, C., BEARD, D., BEARD, J. W., (1961): J. Nat. Cancer Inst., 26; p. 357-388.

HEINE, U., de THE, G., BEARD, D., BEARD, J. W., (1963): J. Nat. Cancer Inst., 30; p. 817.

HIRST, G. K., (1942a): J. Exp. Med., 75; p. 49.

HIRST, G. K., (1942b): J. Exp. Med., 76; p. 195.

HOLMES, J. R., (1958): J. Comp. Path., 68; p. 439.

HOLMES, J. R., (1959): J. Comp. Path., 69; p. 385.

HOLMES, J. R., (1961): J. Comp. Path., 71; p. 20-27.

HOLMES, J. R., (1963): J. Comp. Path., 73; p. 136.

~~HORNE, R. W.~~,

HUEBNER, R. J., (1961): Harvey Lectures, 56; p. 45-62.

HUEBNER, R. J., ROWE, W. P., TURNER, H. U., LANE, W. T., (1963): Proc. Nat. Acad. Sci., 50; p. 379-389.

INMAN, D. R., WOODS, D. A., NEGRONI, G., (1964): Brit. Med. J., April 11; p. 929-931.

JACOBSON, W., WEBB, M., (1952): Exp. Cell Res., 3; p. 1963.

JAMES, J. A., ASHWORTH, C. T., (1961): Am. J. Path., 38; p. 515.

JUNGHERR, E., (1952): in "Diseases of Poultry", p. 453-507: ed., H. E. Biester, L. H. Schwarte. State College Press, Ames, Iowa.

JUNGHERR, E., LANDAUER, W., (1938): Storrs. Agric. Exp. Sta. Bull., 222.

KARNOVSKY, M. J., (1961): J. Biophys. Biochem. Cytol., 11; p. 729.

KARRER, H. E., (1960): J. Ultrastruc. Res., 4; p. 360-371.

KAY, D., (1961): in "Techniques for Electron Microscopy": ed., D. Kay. Blackwell Scientific Publications, Ltd.

KEOGH, E. V., (1938): Brit. J. Exp. Path., 19; p. 1-9.

KOPAC, M.J. (1955): Int. Rev. Cytol., 4; p. 1-26.

KURNICK, N. B., (1949): J. Gen. Physiol., 33; p. 243.

KURNICK, N. B., (1950): Exp. Cell Res., 1; p. 151.

KURNICK, N. B., (1955): Int. Rev. Cytol., 4; p. 221.

KURNICK, N. B., MIRSKY, A. E., (1949): J. Gen. Physiol., 33; p. 265.

KURNITZ, M., (1950): J. Gen. Physiol., 24; p. 15.

LAGERLÖF, B., (1960): Acta. Pathol. Microbiol. Scand., 49, Suppl. 138; pp. 7-19, 344-372, 438-479.

LINDEGREN, C. C., (1951): Exp. Cell Res., 2; p. 275.

LISON, L., (1936): in "Histochemie Animale". Gauthier-Villars, Paris.

LISON, L., (1953): in "Histochemie et Cytoplasmie Animales". Gauthier-Villars, Paris.

LORING, H. S., CARPENTER, F. H., ROLL, P. M., (1947): J. Biol. Chem., 169; p. 601.

LUFT, J. H., (1956): J. Biophys. Biochem. Cytol., 2; p. 799.

LUFT, J. H., (1959): Anat. Rec., 133; p. 305.

MacPHERSON, I., WILDY, P., STOKER, M., HORNE, R. W., (1961); Virology, 13; p. 146-149.

McCULLOCH, E. A., HOWATSON, A. F., SIMINOVITCH, L., AXELRAD, A. A., HAM. A. W., (1959): Nature, 183; p. 1535.

MANNWEILER, K., BERNHARD, W., (1958): Bull. Cancer, 45; p. 223-236.

MAYOR, H. D., JORDAN, L. E., (1962): Virology, 16; p. 325-333.

MAZIA, D., BREWER, P. A., ALFERT, M., (1953): Biol. Bull., 104; p. 69.

MEREKALOVA, Z. I., (1961): Voprosy Virusol, 2; p. 219-223.

MICHAELIS, L., (1947): Cold Spring Harbor Symp., 12; p. 13.

MILLONIG, G., (1961): J. Biophys. Biochem. Cytol., 2; p. 736.

MOLONEY, J. B., (1960): J. Nat. Cancer Inst., 24; p. 933-951.

MOMMAERTS, E. B., SHARP, D.G., ECKERT, E. A., BEARD, D., BEARD, J. W., (1954): J. Nat. Cancer Inst., 4; p. 1011-1025.

MOSCONA, A., (1952): Exp. Cell Res., 3; p. 535.

MURRAY, J. A., BEGG, A. M., (1930): Sci. Rep. Imp. Cancer Res., Fd., 9; p. 1-13.

NEGRONI, G., (1963): Adv. Cancer Res., 7; p. 515-552.

NEGRONI, G., (1964): Brit. Med. J., April 11; p. 927-929.

NEGRONI, G., DOWMASHKIN, R. R., CHESTERMAN, F. C., (1959): Brit. Med. J., p. 1359.

NELSON, F., WALTER, W. G., PRICKETT, C. O., (1946): Poultry Sci., 25; p. 501-508.

NEWMAN, S. B., BORYSKO, E., SWERDLOW, M., (1949): J. Res. Nat. Bur. Stand., 42; p. 183.

OLSON, C., (1941): Cancer Res., 1; p. 384-392.

PAK POY, R. K. F., ROBERTSON, J. S., (1957): J. Biophys. Biochem. Cytol., 3; p. 183-192.

PALADE, G. E., (1952): J. Exp. Med., 95; p. 285.

PARSONS, D. F., BEAUDREAU, G. S., BECKER, C., BONAR, R. A., BEARD, J. W., (1959): Acta. Virio. Intern. contra Cancrum, 15; p. 826-831.

PAUL, J., (1959): in "Cell and Tissue Culture". E.& S. Livingstone, Ltd., Edinburgh and London.

PEACHEY, L. D., (1958): J. Biophys. Biochem. Cytol., 4; p. 233.

PIRAINO, F., OKAZAKI, W., BURMESTER, B. R., FREDRICKSON, T. N., (1963): Virology, 21; p. 396-401.

POLLISTER, A. W., RIS, H., (1957): Cold Spring Harbor Symp., 12; p. 147.

PRINCE, A. M., (1958): J. Nat. Cancer Inst., 20; p. 147-158.

PUGH, L. P., (1927): Vet. Rec., 7; p. 189.

REYNOLDS, E. S., (1963): J. Cell Biol., 17; p. 208.

RHODIN, J. A. G., (1962): in "Renal Disease", p. 117-156: ed., D. A. K. Black. Blackwell Scientific Publications, Oxford.

RHODIN, J. A. G., (1963a): in "Diseases of the Kidney", p. 10-55: ed., M. B. Stauss, L. G. Welt. Little, Brown & Co., Boston.

RHODIN, J. A. G., (1963b): in "An Atlas of Ultrastructure". W. B. Saunders, Co., Philadelphia and London.

RILEY, J. T., WROBLEWSKI, F., (1960): Virology, 14; p. 286-287.

ROBERTSON, J. D., (1958): J. Biophys. Biochem. Cytol., 4; p. 39.

ROMANOFF, A. L., (1960): in "The Avian Embryo, Structural and Functional Development". The Macmillan, Co., New York and London.

ROTHE-MEYER, A., ENGELBRETH-HOLM, J., (1933): Acta. Pathol. Microbiol. Scand., 10; p. 380-427.

ROUILLER, C., HAGUENAU, F., GOLDE, A., LACOUR, F., (1956): Bull. Cancer, 43; p. 10-32.

ROUS, P., MURPHY, J. B., (1912): J. Amer. Med. Assn., 58; p. 1938.

RUBIN, H., (1955): Virology, 1; p. 445-473.

RUBIN, H., (1960): Proc. Nat. Acad. Sci. U.S., 46; p. 1105-1119.

SABATINI, D. D., BERSCH, K. G., BARRNETT, R. J., (1962): "Fifth International Congress for Electron Microscopy, Philadelphia", Electron Microscopy, vol. 2; p. L. 3: ed., S. B. Breese, Jr.

SAMPAIO, M. M., (1956): Anat. Rec., 124; p. 501-518.

SARMA, P. S., TURNER, H. C., HUEBNER, R. T., (1964): Virology, 23; p. 313-321.

SCHWARTZ, S. O., SCHOOLMAN, H. M., SZARTO, P. B., (1956): Cancer Res., 16; p. 559-564.

SEVER, J. L., (1962): J. Microbiol., 88; P. 320-329.

SHARP, D. G., ECKERT, E. A., BEARD, D., BEARD, J. W., (1952): J. Bacteriol., 63; p. 151-161.

SHARP, D. G., BEARD, J. W., (1954): Biochem. et Biophys. Acta., 14; p. 12-17.

SHARPLESS, G. R., DEFENDI, J., COX, H. R., (1958): Proc. Soc. Exp. Biol. Med., 97; p. 755-757.

SILLER, W., (1964): Personal communication.

SOROKIN, S., (1962): J. Cell Biol., 15; p. 363.

STEWART, S. E., EDDY, B. E., GOCHENOUR, A. M., BONGESE, N. G., GRIBBS, G. E., (1957a): Virology, 3; p. 380.

STEWART, S. E., EDDY, B. E., GOCHENOUR, A. M., HAAS, V. H., BONGESE, H. G., (1957b): Ann. N.Y. Acad. Sci., 68; p. 419.

STOKER, M. G. P., (1959): Virology, 8; p. 250-261.

STUBBS, E. L., FURTH, J., (1935): J. Exp. Med., 61; p. 593-615.

TEMIN, H. M., RUBIN, H., (1958): Virology, 6; p. 669-688.

THORELL, B., (1958a): Zentr. Allgem. Pathol. u Pathol. Anat., 98; p. 314-315.

THORELL, B., (1958b): Proc. 7th Intnat. Congr. Intnat. Soc. Haematology, Rome, vol. 3; p. 582.

TOOLAN, H. W., (1954): Cancer Res., 14; p. 660-666.

TRENTIN, J. J., YUBE, Y., TAYLOR, G., (1962): Proc. Am. Assoc. Cancer Res., 3; no. 4; Abstr. no. 279.

VIGIER, P., (1959): Virology, 8; p. 41-59.

VOGT, P. K., RUBIN, H., (1963): Virology, 19; p. 92-104.

WATSON, M. L., (1958): J. Biophys. Biochem. Cytol., 4; p. 475.

WATSON, D. H., RUSSEL, W. C., WILDY, P., (1963): Virology, 19; p. 250-261.

WILDY, P., RUSSELL, W. C., HORNE, R. W., (1960): Virology, 12; p. 204.

ZEIGEL, R. G., (1961): J. Nat. Cancer Inst., 26; p. 1011-1041.

ZETTERQVIST, H., (1956): Ph.D. Thesis, Stockholm.

ACKNOWLEDGEMENTS

I am grateful to Dr. John Carr for the supervision of this work, and to Dr. John Campbell for his many valuable suggestions and criticisms.

I am also grateful to the British Empire Cancer Campaign for making laboratory facilities available to me at the Poultry Research Centre.

This work was undertaken while employed by the British Empire Cancer Campaign.

APPENDIX

FIXATIVES

PALADE'S BUFFERED OSMIUM TETROXIDE (Palade, 1952)

Buffer: (Stock Solution)	Sodium Veronal (Sodium barbitone)	= 2.89 gms)
	Sodium Acetate (Anhydroos)	= 1.15 gms)

Make up in 100 mls distilled water

Osmium Tetroxide: (Stock Solution)	Dissolve 1 gm. of OsO_4 in 50 mls of distilled water	
Fixative:	2% OsO_4	=12.5 mls
	Veronal acetate buffer	= 5.0 mls
	distilled water	= 2.5 mls
	0.1 N-HCl	= 5.0 mls

(Final p.H. should be adjusted to 7.3 - 7.5. Stock solutions are stable for several weeks if kept at 4°C.)

ZETTERQVIST'S BUFFERED ISOTONIC OSMIUM TETROXIDE (Zetterqvist, 1956)

Buffer: (Stock Solution)	Sodium veronal	= 2.94 gms
	Sodium acetate	= 1.94 gms

Make up to 100 mls with distilled water

Ringers solution: (Stock solution)	Sodium chloride	= 8.05 gms
	pottasium chloride	= 0.42 gms
	calcium chloride	= 0.18 gms

Make up to 100 mls with distilled water

Fixative:	2% OsO_4	= 25 mls
	Veronal acetate buffer	= 10 mls
	Ringers solution	= 3.4 mls
	0.1 N-HCl	=11.0 mls

(The fixative is prepared in the same way as Palade's fixative, and thep.H. adjusted to 7.2 - 7.4 with the N-HCl)

CAULFIELD'S BUFFERED OSMIUM TETROXIDE WITH SUCROSE (Caulfield, 1957)

Buffer: (Stock solution)	Sodium Veronal	= 2.94 gms
	Sodium Acetate	= 1.94 gms

Make up to 100 mls with distilled water

Fixative:	2% Osmium Tetroxide	=12.5 mls
	Veronal acetate buffer	= 5.0 mls
	Distilled water	= 2.5 mls
	N/10-HCl	= 5.0 mls

(p.H. is adjusted to 7.4 with N-HCl. 0.045 gms of sucrose is added per ml. of fixative)

LUFT'S BUFFERED PERMANGANATE (Luft, 1956)

Buffer: (Stock Solution)	Prepare veronal acetate buffer in the same way as above.	
(Stock Solution)	Potassium permanganate dissolve in 100 mls of water	= 1.2 gms
Fixative:	Potassium permanganate solution	=12.5 mls
	Veronal acetate buffer	= 5.0 mls
	Distilled water	= 2.5 mls
	N-HCl	= 5.0 mls

GLUTARALDEHYDE (Sabatini et al, 1962)

Buffer; (Stock Solution)	Sodium veronal	=1.47 gms
	Sodium acetate	=0.97 gms
	Sucrose	=11.25gms
	Calcium chloride	= 0.05gms

Dissolve in 200 mls of distilled water and titrate to a p.H. of 7.4 with N-HCl. Make up to 250 mls with distilled water.

Fixative:	Glutaraldehyde	= 5.0 mls
	Veronal acetate buffer	=95.0 mls

This fixative is stable for several months if it is kept at 4°C.

DEHYDRATION OF ELECTRON MICROSCOPE SPECIMENS

Pieces of tissue were dehydrated according to the following schedule:-

40% alcohol	15 minutes
60% alcohol	15 minutes
80% alcohol	15 minutes
90% alcohol	15 minutes
100% alcohol (2 changes)	15 minutes
100% epoxy propane	5 minutes

for araldite) and maraglas)

EMBEDDING MIXTURES

ARALDITE (Glauert and Glauert, 1958)

Araldite M (resin)	10.0 mls
Hardener 964B	10.0 mls
Dibutyl phthalate	1.0 mls
Accelarator	0.5 mls

Specimens were placed in this mixture, in gelatine capsules, and the monomer polymerised at 60°C for 48 hours. No 'washes' in araldite were carried out before polymerisation, since the least viscous araldite mixture (giving the best penetration) was fresh monomer, heated to 60°C.

MARAGLAS (Freeman and Spurlock, 1962)

Maraglas 655	6.0 mls
Maraglas Cardolite NO-513	4.0 mls
Benzyldimethylamine	0.2 mls

Tissues were placed in this embedding mixture, and then the monomer was polymerised by heat at 60°C. for 48 hours.

DURCOPAN (Fluka, A.G., 1963)

(Tissues were dehydrated with acetone in a schedule like that used for araldite and alcohol).

Epoxy resin	10.00 mls
Hardener 964	10.00 mls
Accelerator 964	0.20 mls
Dibutylphthalate	0.15 mls

EMBEDDING PROCEDURE

1. The dehydrated tissues were placed in the embedding mixture, without the accelerator, for one hour at room temperature.

2. The embedding mixture was changed for one with the accelerator, and left for one hour at 50°C.

3. A final change of embedding mixture was made, and the tissues left at 50°C for 48 hours.

ELECTRON MICROSCOPE STAINS

Standard techniques were used for all the lead, the phosphotungstic and phosphomolybdic acid stains.

POTASSIUM PERMANGANATE (Luft, 1956)

The same mixture was used for staining as that described for staining on page 2 of the appendix. Sections were floated on a fresh solution for 1-5 minutes.

URANYL ACETATE AQUEOUS SOLUTION (Brodly, 1959)

The stain was prepared by making a saturated aqueous solution, and then clarifying the solution by centrifugation. Sections were stained for half an hour to two hours.

URANYL ACETATE 50/50 WATER / ALCOHOL SOLUTION Gibbons and Grimstone)

Same preparation as above.

Staining time, 10 - 15 minutes.

Uranyl acetate (100% alcohol)

Same preparation as above solution.

Sections were stained after 10 - 15 minutes,

All the sections stained in uranyl acetate solutions were thoroughly washed in the appropriate solvent, to remove any non-specific precipitation of uranyl acetate.

LIGHT MICROSCOPY TECHNIQUES

These were all according to the standard references.

METHOD OF PARTIALLY PURIFYING VIRUS

This technique was similar to that described by Bather (1954). Tissue extracts were treated to a series of high and low centrifugation cycles according to the following schedule:-

1) Agreous tumour homogenates were centrifuged at 1000 g for 15 minutes
2) The precipitate was discorded and the supernatant decanted and centrifuged for 1 hour at 16000 g.
3) The precipitate obtained from this treatment was resuspended in about 10 mls of Pringus solution and the low and high centrifugation cycle repeated.
4) After two high/low cycles of centrifugation the pellet was resuspended in the appropriate solution: this was the final virus preparation.

TISSUE CULTURE

All the techniques used for the preparation of tissue culture cells can be found in "Cell and Tissue Culture" by Dr. John Paul. However, petri dish cultures were kept in chambers constructed so that cells could be kept in a CO_2 atmosphere in an incubator at 37°C. The boxes were made of perspex 12" x 10" and had detachable lids of ⅜" glass. The lids rested on a thin layer of foam rubber, so that when they were clamped tight the boxes were fairly air-tight. A 5% CO_2/Air mixture could then be introduced by one of two taps. See plate 42, page XV of Appendix.

PLATE 42